(54) **Recombinant birnavirus vaccine**

Rekombinanter Birnavirusimpfstoff

Vaccin recombinant du birnavirus

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(56) References cited:
WO-A-95/26196

• **E.MUNDT AND V.N.VAKHARIA: "Synthetic
transcripts of double-stranded Birnavirus
genome are infectious" PROCEEDINGS OF THE
NATIONAL ACADEMY OF SCIENCES OF USA.,
vol. 93, October 1996, WASHINGTON US, pages
11131-11136, XP002044624**

• **E.MUNDT ET AL.: "Identification of a novel viral
protein in infectious bursal disease
virus-infected cells" JOURNAL OF GENERAL
VIROLOGY, vol. 76, no. 2, February 1995,
READING GB, pages 437-443, XP002044625**

• **E. MUNDT ET AL.: "VP5 of Infectious Bursal
Disease Virus is not essential for virus
replication in cell culture" JOURNAL OF
VIROLOGY, vol. 71, no. 7, July 1997, pages
5647-5651, XP002044626**

• **MURPHY ET AL: 'PRODUCTION AND LEVEL OF
GENETIC STABILITY OF AN INFLUENZA A
VIRUS TEMPERATURE-SENSITIVE MUTANT
CONTAINING TWO GENES WITH TW
MUTATIONS' INFECTION AND IMMUNITY vol.
37, no. 1, 1982, pages 235 - 242**

• **Mundt E: An Abstract circulated at the Annual
Meeting of the German Virological Society 1997
(Hamburg, 10-13 March 1997)**

• **Mundt E: A poster presented at the Annual
Meeting of the German Virological Society 1997
(Hamburg, 10-13 March 1997)**

• **Mundt I: An Abstract in the Annual Report 1996
of the German Federal Research Institute for
virus diseases in animal, published April 1997**

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Description

[0001] The present invention is concerned with a birnavirus mutant, a vaccine comprising this mutant, a method for determining birnavirus infection in an animal, as well as with a test kit for carrying out this method.

[0002] ·Infectious bursal disease virus (IBDV) and Infectious pancreatic necrosis virus (IPNV) are members of the Birnaviridae family. Viruses in this family have a very similar genomic organisation and a similar replication cycle. The genomes of these viruses consist of 2 segments (A and B) of double-stranded (ds) RNA. The larger segment A encodes a polyprotein which is cleaved by autoproteolysis to form mature viral proteins VP2, VP3 and VP4 (Hudson, P.J. et al, Nucleic Acids Res., 14, 5001-50012, 1986; Dobos P., Annual review of fish diseases 5, 25-54, 1995). VP2 and VP3 are the major structural proteins of the virion. VP2 is the major host-protective immunogen of birnaviruses, and contains the antigenic regions responsible for the induction of neutralising antibodies. The VP4 protein appears to be a virus-coded protease that is involved in the processing of a precursor polyprotein of the VP2, VP3 and VP4 proteins. The larger segment A possesses also a second open reading frame (ORF), preceding and partially overlapping the poly-protein gene. This second open reading frame encodes a protein VP5 of unknown function that is present in IBDV infected cells (Mundt, E. et al., J. Gen. Virol., 76, 437-443, 1995).

[0003] The smaller segment B encodes VP1, a 90 kDa multifunctional protein with polymerase and capping enzyme activities (Spies, U. et al., Virus Res., 8, 127-140, 1987 and Spies, U. et al., J. Gen. Virol., 71, 977-981, 1990; Duncan R. et al., Virology 181, 541-552, 1991).

[0004] For IBDV, two serotypes exist, serotype 1 and 2. The 2 serotypes may be differentiated by virus neutralisation (VN) tests. Furthermore, subtypes of serotype 1 have been isolated. These so-called "variant" viruses of serotype 1 can be identified by cross-neutralisation tests (Diseases of Poultry, 9th edition, 1991, Wolfe Publishing Ltd, ISBN 0 7234 1706 7, Chapter 28, P.D. Lukert and Y.M. Saif, 648-663), a panel of monoclonal antibodies (Snyder, D.B. et al., Arch. Virol., 127, 89-101. 1992.) or RT-PCR (Jackwood, D.J., Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 155-161, 1994). Some of these sub-types of serotype 1 of IBDV have been described in literature for example: Classical, Variant-E, GLS, RS593 and DS326 strains (Van Loon, et al. Proceedings of the International symposium on infectious bursal disease and chicken infectious anaemia, Rauischholzhausen, Germany, 179-187, 1994).

[0005] Infectious Bursal disease (IBD), also called Gumboro disease, is an acute, highly-contagious viral infection in chickens that has lymphoid tissue as its primary target with a selective tropism for cells of the bursa of Fabricius. The morbidity rate in susceptible flocks is high, with rapid weight loss and moderate mortality rates. Chicks that recover from the disease may have immune deficiencies because of the destruction of the bursa of Fabricius which is essential to the defence mechanism of the chicken. The IBD-virus causes severe immunosuppression in chickens younger than 3 weeks of age and induces bursal lesions in chicks up to 3 months old.

[0006] For many years the disease could be prevented by inducing high levels of antibodies in breeder flocks by the application of an inactivated vaccine, to chickens that had been primed with attenuated live IBDV vaccine. This has kept economic losses caused by IBD to a minimum. Maternal antibodies in chickens derived from vaccinated breeders prevents early infection with IBDV and diminishes problems associated with immunosuppression. In addition, attenu-ated live vaccines have also been used successfully in commercial chicken flocks after maternal antibodies had de-clined.

[0007] Recently, very virulent strains of IBDV have caused outbreaks of disease with high mortality in Europe. The current vaccination programs failed to protect chicks sufficiently. Vaccination failures were mainly due to the inability of live vaccines to infect the birds before challenge with virulent field virus.

[0008] Eradication of the disease by other preventative measures than vaccination has not been feasible, because the virus is widely spread and because with currently administered live attenuated or inactivated IBDV vaccines it is not possible to determine whether a specific animal is infected with an IBDV field virus or whether the animal was vaccinated with an IBDV vaccine. In order to be able to start an eradication control programme for IBDV it is highly desirable that the possibility exists to discriminate between animals vaccinated with an IBDV vaccine and those infected with a field virus so as to be able to take appropriate measures, i.e. remove infected flocks, to reduce spreading of the virulent field virus. The introduction of, for example, a serologically identifiable marker can be achieved by introducing a mutation in genes encoding non-essential (glyco)proteins of the IBDV which still give rise to the production of anti-bodies in an infected host animal. A marker vaccine for Aujeszky's disease and companion diagnostic tests have proven their practical value in the control of this disease. Whereas such control programs for other viral infectious diseases in animals are under development, until the present invention a vaccine based on an IBDV vaccine strain which would fit in IBDV control programs has not been described yet.

[0009] Mundt and Köllner (Annual Meeting of the German Federal Research Institute for virus diseases in animals, March 1997)) disclose the construction of an IBDV mutant that fails to express a VP5 protein as a result of a genetically engineered mutation in the start codon of the VP5 gene. This mutation comprises the substitution of one nucleotide in the start codon of the VP5 gene.

[0010] The present invention provides a birnavirus mutant as defined in the claims which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome.

[0011] Preferably, the birnavirus mutant is an IBDV mutant or an IPNV mutant, the IBDV mutant being most preferred, in particular an IBDV mutant derived from a serotype 1 IBD virus is provided by the present invention.

[0012] It is demonstrated that an IBDV mutant that is not able to produce a VP5 protein is still able to infect poultry and to replicate in the infected host animals in vivo, i.e. evidence is provided that the gene encoding the VP5 protein is a non-essential gene. Example 3 and 4 show that VP5⁻ IBDV can be re-isolated from organs of animals infected with the IBDV mutant and that the IBDV mutant induces a protective immune response in the infected animals.

[0013] Moreover, it has been established herein that part of the normal anti-IBDV immune response in poultry is directed to the VP5 region. This is rather surprising as the VP5 protein is considered to represent a non-structural viral protein (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) and the immune response in an animal against a viral pathogen is usually elicited against the structural (glyco)proteins of the virus. These findings make the IBDV mutant and other birnavirus mutants according to the present invention a suitable vaccine candidate for a marker vaccine. Such a marker vaccine provides the possibility to determine whether animals are infected with a wild-type birnavirus, e.g. IBDV, or with a vaccine virus.

[0014] Additionally, it has been found that the VP5 protein is involved in the expression of virulence of the birnaviruses, in particular of IBDV, and that the inability of the virus mutants to produce the native VP5 protein leads to an attenuation of the virus.

[0015] With the term "which is not able to produce a native VP5 protein" is meant that the birnavirus mutant produces a polypeptide that can be distinguished by serological tests from the native VP5 protein, or does not produce a VP5 protein at all. For example, in the former case, the birnavirus mutant produces only a fragment of the native birnavirus VP5 protein which lacks one or more immunogenic epitopes.

[0016] Preferably, the birnavirus mutant according to the invention produces no VP5 protein upon infection of a host cell.

[0017] As described above, the genomic organisation of the birnaviruses is well established: the IBDV and IPNV genome comprises a large segment A and a smaller segment B. The segment A of IBDV comprises a large open reading frame (ORF) encoding a polyprotein of about 110 kDa (VP2-VP4-VP3). The gene encoding the VP5 protein is identified in the prior art, and defined herein, as the small ORF on segment A of the birnavirus genome which precedes and partially overlaps the polyprotein encoding ORF (Bayliss et al., J. Gen. Virol. 71, 1303-1312, 1990; Spies et al., J. Gen. Virol. 71, 977-981, 1990; Havarstein L.S. et al., J. Gen. Virology 71, 299-308; 1990; Dobos et al., 1995, supra; Figures 1-3 herein and SEQ ID No.'s 1-7). The mutation introduced in the VP5 gene is such that it does not prevent the expression of the polyprotein.

[0018] SEQ ID No. 1 comprises the full length cDNA nucleotide sequence of segment B of IBDV strain P2, as well as the amino acid sequence of the VP1 protein encoded by segment B (see also SEQ ID. No. 2). SEQ ID No. 3 and 5 depict the full length cDNA sequence of segment A of IBDV strain D78 and the coding region of the VP5 protein and the polyprotein, respectively. SEQ ID 3 and 4 also show the amino acid sequence of the D78 VP5 protein. SEQ ID No. 5 and 6 show the amino acid sequence of the polyprotein VP2-VP4-VP3 of D78. SEQ ID No. 7 shows the 5'-end of segment A of strain D78, including the mutations introduced in the VP5 coding region. SEQ ID No. 8 shows the nucleotide sequence of segment B of strain D78 and the amino acid sequence of the D78 VP1 protein. The genomic organisation of both segments is also shown in Figure 1.

[0019] The ORF coding for VP5 is conserved in all hitherto published segment A sequences. The IBDV ORF encodes 145 amino acids resulting in a calculated molecular mass of 16.5 kDa. The nucleotide sequence of the ORF encoding the VP5 protein of IBDV strain D78 used herein is shown in SEQ ID No. 3 and 4. Natural variations may exist between individual IBDV isolates. These natural variations result from small differences in the genomes of these viruses. The nucleotide sequence of the segment A, including the nucleotide sequence of the VP5 gene for many IBDV isolates have been described in the prior art (Vakharia et al., Avian Diseases 36, 736-742, 1992; Bayliss et al., J. Gen. Virol. 71, 1303-1314, 1990; Hudson et al., Nuc. Acid Res. 14, 5001-5012, 1986; Schnitzler et al., J. Gen. Virol. 47, 1563-1571, 1993; Kibenge et al., J. Gen. Virol. 71, 569-577, 1990 and Virology 184, 437-440, 1991; Mundt et al., Virology 209, 10-18, 1995; Lana et al., Virus Genes 6, 247-259, 1992; Vakharia et al., Virus Res. 31, 265-273, 1994; Brown et al., Virus Res. 40, 1-15, 1996). The amino acid sequence of the VP5 protein from serotype I IBDV strains display a homology of at least 95% with the VP5 amino acid sequence shown in SEQ ID No. 3 and 4, whereas the homology between serotype II VP5 sequence and the amino acid sequence shown in SEQ ID No. 3 and 4 is at least 75%. Therefore, a preferred IBDV mutant according to the present invention is an IBDV mutant wherein the mutation is introduced in the VP5 gene having a homology of at least 75%, in particular at least 95% on the amino acid sequence level with the VP5 amino acid sequence shown herein.

[0020] Preferably an IBDV mutant according to the present invention is derived from any of the classical or variant (e.g. variant E or GLS) IBDV vaccine strains, such as those currently used in the field. Such suitable IBDV strains include the IBDV vaccine strains present in the commercially available vaccines: D78, PBG 98, LZ 228E, 89-03 (Intervet

International B.V.), Bursine 2 (Fort Dodge Animal Health) and S 706 (Rhone Mérieux).

[0021] A particular preferred IBDV mutant according to the invention is derived from the D78 strain comprising a VP5 gene encoding a protein having the amino acid sequence shown in SEQ ID No. 3 and 4.

[0022] Alternatively, the parent birnavirus strain for the virus mutant according to the invention is a virulent birnavirus field strain. It is found herein that the VP5 protein is a factor associated with virulence, and that the absence of the native VP5 protein in a birnavirus results in an attenuated form of the virus.

[0023] Preferably the invention provides a birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the part of the VP5 gene which does not overlap with the large ORF encoding the polyprotein.

[0024] In particular, the birnavirus mutant according to the invention comprises a mutation in the 5'-end of the VP5 gene spanning nucleotides 1-30, preferably 1-20, more preferably 1-10. Most preferred is an birnavirus mutant having a mutation in nucleotides 1-3 of the VP5 gene.

[0025] A mutation is understood to be a change of the genetic information in the VP5 gene with respect to the genetic information present in this region of the genome of naturally occurring birnavirus producing native VP5 protein. The mutation is, for example, a nucleic acid substitution, deletion, insertion or inversion, or a combination thereof.

[0026] In a preferred embodiment of the present invention a birnavirus mutant is provided wherein the mutation is a substitution of one or more nucleotides. In particular, a nucleic acid substitution is introduced in the start codon, as a result of which the new codon encodes an amino acid different from methionine or represents a stop codon, preferably the nucleic acid substitution comprises at least two of the nucleotides of the start codon.

[0027] A further birnavirus mutant according to the invention comprises a substitution of one or more nucleotides in a codon(s) different from the start codon resulting in one or more stop codons, preferably in the 5'-end of the VP5 gene as defined above, if desired in addition to a substitution in the start codon as described above. Preferably, the birnavirus mutant comprises a stop codon in this region of the VP5 gene in each of the three reading frames.

[0028] Such a preferred birnavirus mutant may be an IBDV mutant having a mutation in the start codon, the fourth and the sixth codon of the VP5 gene, preferably resulting in the mutated codons shown in SEQ ID No. 7 and Figure 3.

[0029] Alternatively, a birnavirus mutant is provided wherein the mutation is a deletion. In particular, the deletion comprises less than 20, less than 10 or less than 5 nucleotides. Preferably, the deletion comprises a total number of nucleotides not dividable by three, resulting in a shift of the reading frame.

[0030] Preferably the deletion comprises one or more nucleotides of the start codon of the VP5 gene.

[0031] In an alternative embodiment of the present invention a bimavirus mutant is provided wherein the mutation comprises the insertion of a heterologous nucleic acid sequence in the birnavirus genome. A heterologous nucleic acid sequence is a nucleic acid sequence normally not present at the specific insertion site of the particular virus species.

[0032] The heterologous nucleic sequence to be incorporated into the birnavirus genome is a nucleic acid fragment which either encodes a polypeptide or is a non-coding sequence. The nucleic acid fragment can be derived from any source, e.g. viral, eukaryotic, prokaryotic or synthetic, including oligonucleotides suitable for the interruption of the expression of the VP5 gene.

[0033] A suitable oligonucleotide for the interruption of the VP5 expression may comprise three translational stop codons in each of the possible reading frames in both directions, in addition to one or more appropriate restriction enzyme cleavage sites useful for the insertion of a second heterologous nucleic acid sequence. The length and nucleotide sequence of such a non-coding heterologous nucleic acid sequence is not critical, but preferably varies between 8-50 nucleotides.

[0034] In a further embodiment of the present invention a bimavirus mutant is provided which can be used not only for the preparation of a vaccine against infection by a specific birnavirus, but also against other poultry or fish infectious diseases. For example, a vector vaccine based on such an IBDV mutant offers the possibility to immunise against other avian pathogens by the expression of antigens of these avian pathogens within infected cells of the immunised host. Such an IBDV vector according to the present invention can be obtained by inserting a heterologous nucleic acid sequence encoding a polypeptide heterologous to the IBDV in the VP5 gene as defined herein.

[0035] The heterologous nucleic acid sequence may encode an antigen of an avian pathogen such as Newcastle disease virus, Infectious bronchitis virus, Marek's disease virus, avian encephalomyelitis virus, avian reovirus, avian influenza virus, chicken anaemia virus, Salmonella spp., E.coli, and Eimeria spp.

[0036] Furthermore, an IBDV mutant according to the invention comprises in addition to the mutation in the VP5 gene, a mutation in the VP2 gene, wherein this gene expresses a chimeric protein comprising neutralising epitopes of more than one antigenic type of IBDV (e.g. classic, Variant-E and/or GLS). Preferably, such a mutant comprises the relevant protective VP2 epitopes of a variant GLS strain and classic strain. In particular, the mutated VP2 gene is a GLS VP2 gene comprising a nucleic acid sequence fragment encoding the B69 epitope. The construction of such a mutated VP2 genes is described in Snyder et al., Avian Diseases 38, 701-707, 1994. Furthermore, nucleic acid sequences encoding polypeptides for pharmaceutical or diagnostic applications, in particular immuno-modulators such as lymphokines, interferons or cytokines, may be incorporated into the VP5 gene. The heterologous nucleic acid sequence may also encode a screenable marker, such as E. coli β-galactosidase or E. coli β-glucuronidase.

[0037] The construction of birnavirus mutants, in particular of IBDV mutants according to the present invention can be achieved by means of the recently established infectious cRNA system for IBDV (Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996). This reverse genetics system opens the possibility to introduce mutations in the RNA genome of the IBD virus, in particular in the VP5 gene. The most important step in this reverse genetics system is to provide full length cDNA clones of the segments A and B of IBD virus. cDNA constructs comprising the segment A or B, including the nucleotides of the 5'- and 3'- ends of both these segments can be generated according to the method described by Mundt and Vakharia (1996, supra). Additionally, these constructs comprise a RNA polymerase promoter operably linked to either of the segments. The promoter can be the promoter for the T7, SP6 or T3 polymerase, the T7 promoter being preferred. Mutations can be introduced into the VP5 gene by means of methods generally known in the art for this purpose. In particular, the mutation(s) are introduced by means of site directed mutagenesis.

[0038] For example, in a first step a cDNA fragment is provided comprising at least a substantial part of the VP5 gene. In the next step suitable primer pairs are designed and hybridised with the VP5 sequence containing fragment. The 5'-primer comprises in addition to sequences complementary to the VP5 sequence, nucleotides which harbour the desired mutation, e.g. a mutation which changes the ATG start codon to an AGG (arginine) codon. Moreover, the 5'-primer is provided with an upstream nucleotide sequence representing a suitable restriction enzyme cleavage site which allows the restoring of the complete 5'-end non-coding sequence. Subsequently, the new mutated fragment is amplified using PCR and the new fragment is introduced in the starting sequence by replacing the native nucleic acid sequence using appropriate restriction enzymes. In the next step plus-sense transcripts of the segment A and B are generated in vitro with (T7) RNA polymerease, after which the synthetic transcripts are purified using conventional RNA purification techniques. The recombinant IBDV mutant according to the invention is obtained after transfection of suitable cells (e.g. VERO cells, QM-7 cells or CEC cells) with the synthetic RNA transcripts of both segments of the IBDV genome, if desired in the presence of transfection-enhancing compositions, such as Lipofectin. Finally the recombinant IBDV is harvested from the supernatant of the transformed cells.

[0039] Methods for introducing a mutation in the birnavirus genome are described herein, but are also generally used in the art (Mundt and Vakharia, 1996, supra; Current Protocols in Molecular Biology, eds.: F. M. Ausubel et al., Wiley N.Y., 1995 edition, pages 8.5.1.-8.5.9.)

[0040] Further to the unexpected finding by the present inventors that the VP5 ORF of IBDV is a non-essential region of the IBDV genome, it has also been found that an IBDV mutant according to the present invention is able to induce a protective immune response, i.e. animals immunised with a vaccine comprising the IBDV mutant are protected against virulent challenge. Moreover, it has been found that anti-sera of animals infected with naturally occurring IBDV comprise antibodies directed to the non-structural VP5 protein and that these antisera can be distinguished from anti-sera derived from animals infected with an IBDV mutant according to the present invention. In addition, it has been found that the IBDV mutant as described above is attenuated if compared with the parent IBD virus which is able to produce the native VP5 protein.

[0041] Therefore, another aspect of this invention is a vaccine for use in the protection of animals against birnavirus infection comprising the birnavirus mutant as characterised above, together with a pharmaceutical acceptable carrier or diluent. In particular, the vaccine according to the invention is a vaccine for use in the protection of poultry against infectious bursal disease comprising the IBDV mutant described above.

[0042] The birnavirus mutant according to the present invention can be incorporated into the vaccine as live or inactivated virus.

[0043] A vaccine according to the invention can be prepared by conventional methods such as for example commonly used for the commercially available live- and inactivated IBDV vaccines. Briefly, a susceptible substrate is inoculated with an IBDV mutant according to the invention and propagated until the virus replicated to a desired infectious titre after which IBDV containing material is harvested.

[0044] Every substrate which is able to support the replication of IBD viruses can be used in the present invention, including primary (avian) cell cultures, such as chicken embryo fibroblast cells (CEF) or chicken kidney cells (CK), mammalian cell lines such as the VERO cell line or the BGM-70 cell line, or avian cell lines such as QT-35, QM-7 or LMH. Usually, after inoculation of the cells, the virus is propagated for 3-10 days, after which the cell culture supernatant is harvested, and if desired filtered or centrifuged in order to remove cell debris.

[0045] Alternatively, the IBDV mutant is propagated in embryonated chicken eggs. In particular, the substrate on which these IBD viruses are propagated are SPF embryonated eggs. Embryonated eggs can be inoculated with, for example 0.2 ml IBDV mutant containing suspension or homogenate comprising at least 10^2 TCID$_{50}$ per egg, and subsequently incubated at 37 °C. After about 2-5 days the IBD virus product can be harvested by collecting the embryo's and/or the membranes and/or the allantoic fluid followed by appropriate homogenising of this material. The homogenate can be centrifuged thereafter for 10 min at 2500 x g followed by filtering the supernatant through a filter (100 μm).

[0046] The vaccine according to the invention containing the live virus can be prepared and marketed in the form of a suspension or in a lyophilised form and additionally contains a pharmaceutically acceptable carrier or diluent cus-

tomary used for such compositions. Carriers include stabilisers, preservatives and buffers. Suitable stabilisers are, for example SPGA, carbohydrates (such as sorbitol, mannitol, starch, sucrose, dextran, glutamate or glucose), proteins (such as dried milk serum, albumin or casein) or degradation products thereof. Suitable buffers are for example alkali metal phosphates. Suitable preservatives are thimerosal, merthiolate and gentamicin. Diluents include water, aqueous buffer (such as buffered saline), alcohols and polyols (such as glycerol).

[0047]   If desired, the live vaccines according to the invention may contain an adjuvant. Examples of suitable compounds and compositions with adjuvant activity are the same as mentioned below.

[0048]   Although administration by injection, e.g. intramuscular, subcutaneous of the live vaccine according to the present invention is possible, the vaccine is preferably administered by the inexpensive mass application techniques commonly used for IBDV vaccination. For IBDV vaccination these techniques include drinking water and spray vaccination.

[0049]   Alternative methods for the administration of the live vaccine include in ovo, eye drop and beak dipping administration.

[0050]   In another aspect of the present invention a vaccine is provided comprising the birnavirus mutant in an inactivated form. The major advantage of an inactivated vaccine is the extremely high levels of protective antibodies of long duration that can be achieved.

[0051]   The aim of inactivation of the viruses harvested after the propagation step is to eliminate reproduction of the viruses. In general, this can be achieved by chemical or physical means. Chemical inactivation can be effected by treating the viruses with, for example, enzymes, formaldehyde, β-propiolactone, ethylene-imine or a derivative thereof. If necessary, the inactivating compound is neutralised afterwards. Material inactivated with formaldehyde can, for example, be neutralised with thiosulphate. Physical inactivation can preferably be carried out by subjecting the viruses to energy-rich radiation, such as UV light or γ-rays. If desired, after treatment the pH can be adjusted to a value of about 7.

[0052]   A vaccine containing the inactivated birnavirus mutant can, for example comprise one or more of the above-mentioned pharmaceutically acceptable carriers or diluents suited for this purpose.

[0053]   Preferably, an inactivated vaccine according to the invention comprises one or more compounds with adjuvant activity. Suitable compounds or compositions for this purpose include aluminium hydroxide, -phosphate or -oxide, oil-in-water or water-in-oil emulsion based on, for example a mineral oil, such as Bayol F® or Marcol 52® or a vegetable oil such as vitamin E acetate, and saponins.

[0054]   The vaccine according to the invention comprises an effective dosage of the birnavirus mutant as the active component, i.e. an amount of immunising birnavirus material that will induce immunity in the vaccinated birds against challenge by a virulent virus. Immunity is defined herein as the induction of a significant higher level of protection in a population of birds after vaccination compared to an unvaccinated group.

[0055]   Typically, the live vaccine according to the invention can be administered in a dose of 10^2-10^9 TCID$_{50}$ infectious dose$_{50}$ (TCID$_{50}$) per animal, preferably in a dose ranging from $10^{5.0}$-$10^{7.0}$ TCID$_{50}$, and an inactivated vaccines may contain the antigenic equivalent of 10^5-10^9 TCID$_{50}$ per animal.

[0056]   Inactivated vaccines are usually administered parenterally, e.g. intramuscularly or subcutaneously.

[0057]   Although, the IBDV vaccine according to the present invention may be used effectively in chickens, also other poultry such as turkeys, guinea fowl and partridges may be successfully vaccinated with the vaccine. Chickens include broilers, reproduction stock and laying stock.

[0058]   The age of the animals receiving a live or inactivated vaccine according to the invention is the same as that of the animals receiving the conventional live- or inactivated IBDV vaccines. For example, broilers (free of maternally derived antibodies-MDA) may be vaccinated at one-day-old, whereas broilers with high levels of MDA are preferably vaccinated at 2-3 weeks of age. Laying stock or reproduction stock with low levels of MDA may be vaccinated at 1-10 days of age followed by booster vaccinations with inactivated vaccine on 6-8 and 16-20 weeks of age.

[0059]   The invention also includes combination vaccines comprising, in addition to the IBDV or IPNV mutant according to the invention, one or more immunogens derived from other pathogens infectious to poultry or fish, respectively.

[0060]   Preferably, the combination vaccine additionally comprises one or more vaccine strains of infectious bronchitis virus (IBV), Newcastle disease virus (NDV), egg drop syndrome (EDS) virus, turkey rhinotracheitis virus (TRTV) or reovirus.

[0061]   In addition to a marker vaccine for birnaviruses, the availability of an appropriate diagnostic test is an essential requirement for the application of a birnavirus eradication control programme. Such a diagnostic test is provided herein and comprises a method for determining IBDV infection in poultry and IPNV infection in fish, i.e. it provides a method for distinguishing an animal in the field vaccinated with a vaccine as described above, from an animal infected with a naturally-occurring IBDV or IPNV.

[0062]   Therefore, the present invention provides a method for the detection of birnavirus infection, in particular for the detection of IBDV infection in an animal comprising the step of examining a sample of the animal for the presence of VP5 antibodies or antigens. The animal is an animal from the field and is in particular an avian species, preferably

a chicken. The sample coming from the animal may be any sample in which IBDV antibodies or antigens are present, e.g. a blood, serum or tissue sample, the serum sample being preferred.

[0063] A preferred method for determining birnavirus infection in an animal is a method for the detection of antibodies against the VP5 protein, comprising the steps of:

(i) incubating a sample suspected of containing anti-birnavirus antibodies, with VP5 antigen,
(ii) allowing the formation of antibody-antigen complex , and
(ii) detecting the presence of the antibody-antigen complex.

[0064] The design of this immunoassay may vary. For example, the immunoassay may be based upon competition or direct reaction. Furthermore, protocols may use solid supports or may use cellular material. The detection of the antibody-antigen complex may involve the use of labelled antibodies; the labels may be, for example, enzymes, fluorescent-, chemiluminescent-, radio-active- or dye molecules.

[0065] Suitable methods for the detection of the VP5 antibodies in the sample include the enzyme-linked immunosorbent assay (ELISA), immunofluorescent test (IFT) and Western blot analysis.

[0066] In an exemplifying ELISA, the wells of a polystyrene micro-titration plate are coated with VP5 antigen. Next, the wells of the coated plates are filled with chicken serum and serial dilutions are made. After incubation, chicken anti-VP5 protein serum antibodies are determined by detecting antibody (monoclonal or polyclonal) with the same specificity as the coated one, but which is labelled (e.g. with biotin). The labelled antibody will occupy the free antigens that have not been occupied by anti-VP5 antibodies in the chicken serum. For example, horse radish peroxidase coupled to avidin may be added and the amount of peroxidase is measured by an enzymatic reaction. If no antibodies against VP5 are present in the chicken serum sample then a maximum absorption is obtained. If the serum contains many antibodies against VP5 then a low absorption is expected. Alternatively, after the incubation with chicken serum, the amount of antibodies present in the serum that bound to the VP5 antigen may be determined directly by using an anti-chicken conjugate followed by the enzymatic reaction.

[0067] In a sandwich ELISA the wells of a polystyrene micro-titration plate can be coated with a monoclonal antibody directed against the VP5 protein. Next, the wells of these coated plates are incubated with VP5 antigen. After the antigen is captured, the wells are filled with the chicken serum and serial dilutions are made. Subsequently, the protocol as described above may be followed. This test can also be carried out by using polyclonal serum against VP5 instead of the coated monoclonal antibodies.

[0068] In another diagnostic test (Western blot analysis), the VP5 antigen (containing) material is subjected to SDS-PAGE. Next, the separated proteins are electroblotted onto nitro-cellulose membrane. Thereafter, the membranes can be cut into lanes and the lanes are incubated with the chicken serum. The presence of VP5 antibodies in the sample can be determined by examination whether antibodies bound to the VP5 antigen, for example by using an anti-chicken conjugate followed by an enzymatic reaction. If antibodies against VP5 are present then a band at about 17 kDa is identifiable.

[0069] The VP5 antigen may be any VP5 protein (fragment) comprising material which allows the formation of the VP5 antigen-VP5 antibody complex. Preferably, the VP5 antigen comprises the expression product of a conventional recombinant host cell or virus, e.g. such as E.coli expressed VP5 (Mundt et al., J. Gen. Virol. 76, 437-443, 1995) or baculovirus expressed protein (Vakharia et al., Vaccine 12, 452-456, 1994; Vakharia et al., J. Gen Virol. 74, 1201-1206, 1993). In a further embodiment of the present invention a diagnostic test kit is provided which is suitable for performing the diagnostic test according to the invention as described above.

[0070] In particular, a diagnostic test kit is provided which comprises in addition to the components usually present, the VP5 antigen (if desired coated onto a solid phase) as the immunological reagent. Other components usually present in such a test kit include, biotin or horseradish peroxidase conjugated antibodies, enzyme substrate, washing buffer etc.

[0071] To determine birnavirus VP5 antigen in a test sample from an animal in the field, VP5-specific antibodies are used as the immunological reagent, preferably fixed to a solid phase. The test sample is added, and after an incubation time allowing formation of the antibody-antigen complex, a second labelled antibody may be added to detect the complex.

„ EXAMPLES

Example 1.

Construction and analysis of recombinant VP5⁻ IBD virus

Construction of full length VP5⁻ clone of IBDV segment A.

[0072] To construct a VP5-negative IBDV, the *Eco*RI site immediately following the 3'-end of the full length cDNA of strain D78 segment A (pUC19FLAD78; Mundt and Vakharia, Proc. Natl. Acad. Sci. USA 93, 11131-11136, 1996) was deleted. An *Eco*RI - *Kpn*I fragment containing the T7 polymerase binding site followed by the complete segment A sequence was excised and inserted into *Eco*RI - *Kpn*I cleaved vector pUC18 after inactivation of the unique *Nde*I within the vector sequence resulting in plasmid pAD78/EK. Thereafter, the genomic region encompassing the initiation codon for VP5 was amplified in two pieces using primers A1F5' and VP5MutR, and VP5MutF and A2R, respectively (see Table 1 for sequence and location of primers). PCR fragments were cloned separately and were subsequently fused via a unique *Afl*II site which had been created by mutations within respective primers (see Fig. 2). An *Eco*RI - *Nde*I fragment containing the T7 polymerase binding site, and the 5'-part of segment A including the introduced mutations was excised and used to substitute the wild-type *Eco*RI - *Nde*I fragment in pAD78/EK to yield plasmid pAD78/VP5⁻. Of the three mutations introduced one altered the initiation methionine codon for VP5 into an arginine codon (Fig. 2).

Table 1: Sequence of oligonucleotide primers used for generating mutant constructs.

ªNucleotide sequence	Orientation	Designation	Nucleotide no.
AGAGAATTC*TAATACGACTCACTATA*GGATACGATCGGTCTGAC	+	A1F5'	1-18
TGGGCCTGTCACTGCTGTCACATGT	-	A2R	716 - 740
CATTGCTCTGCAGTGTGTAGTGAGC	-	A3R	338 - 362
CTACAACGCTATCCTTAAGGGTTAGTAGAG	+	VP5MutF	80 - 109
CTCTACTAACCCTTAAGGATAGCGTTGTAG	-	VP5MutR	80 - 109

a)   Underlined nucleotides denote virus specific nucleotides. T7 promotor sequences are marked in italics. Mutated nucleotides are bold and orientation of the primer is shown for sense (+) and antisense (-). Primer positions are given according to the published sequence of serotype I strain P2 (Mundt et al., Virology 209, 209-218, 1995).

[0073]   Virus recovery from cRNA. For *in vitro* transcription of RNA plasmids pAD78/EK, pAD78/VP5⁻ and pBP2 (Fig. 2) were linearized by cleavage with *Bsr*GI and *Pst*I, respectively. Treatment of linearized DNA, transcription and purification of RNA, and transfection were carried out as described by Mundt and Vakharia (1996, supra) with the exception that secondary CEC were used for the transfection experiments. Three days after transfection a CPE was visible in CEC. Cells were freeze/thawed, centrifuged at 700 x g to eliminate cellular debris, and the resulting super-natants were filtrated through 0.45 μm filters and stored at -20°C. For the transfection experiments full length cDNA clones of segment A of strain D78 capable of expressing (pAD78/EK) or unable to express VP5 (pAD78/VP5⁻) were transcribed into synthetic RNA and cotransfected with segment B full length cRNA into CEC. Resulting virus progeny IBDV/EK and IBDV/VP5⁻ was further characterised.

[0074]   Analysis of transfection progeny by immunofluorescence and Radioimmunoprecipitation assay

(RIPA). VP5 was expressed in E.coli as described in Mundt et al. (J. Gen. Virol. 76, 437-443, 1995). Rabbit monospecific polyclonal anti serum and mouse monoclonal antibodies against VP5 were prepared according to standard protocols. Vero cells infected with IBDV/VP5⁻, IBDV/EK, and non-infected cells , respectively, were incubated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and with anti-VP5 mAb DIE 7, and stained with fluoresceine-conjugated secondary antibodies. Both antisera and the monoclonal antibody recognised IBDV antigens in the cytoplasm of IBDV/EK infected cells. In contrast, whereas the anti-IBDV serum readily detected viral antigens in IBDV/VP5⁻ infected cells, neither the monospecific anti VP5-serum nor the monoclonal anti-VP5 antibody exhibited specific reactivity. None of these immunological reagents reacted with non-infected controls.

[0075] To analyse viral proteins expressed during replication lysates of radioactively labelled CEC infected with IBDV/VP5⁻ (Fig 4, lanes 1-3) and IBDV/EK (Fig. 4, lanes 4-6) were immunoprecipitated with rabbit anti-IBDV serum, rabbit anti-VP5 serum and mAb DIE 7. Non-infected CEC were used as control (Fig. 4, lanes 7-9). IBDV/EK (lane 4) as well as IBDV/VP5⁻ (lane 1) infected CEC showed viral proteins VP2, VP3, and VP4 after precipitation with rabbit anti-IBDV serum. The rabbit anti-VP5 serum (lane 5) and mAb DIE 7 (lane 6) precipitated VP5 with a molecular mass of 21 kDa only from IBDV/EK infected cells. No specific reactivity was detectable in IBDV/VP5⁻ infected CEC after precipitation with rabbit-anti VP5 (lane 2) as well as the VP5 specific mAb DIE 7 (lane 3). Non-infected CEC showed no specific reactivity (lanes 7-9).

[0076] **Replication of IBDV/VP5⁻ in CEC.** To assay replication of IBDV/VP5⁻ in more detail one step growth was analysed (Fig. 5). Confluent secondary CEC were infected with IBDV/EK and IBDV/VP5⁻ with $10^{7.2}$ TCID$_{50}$, respectively. Immediately after overlaying the infected cells with 5 ml growth medium, supernatant from one infected CEC tissue plate of each virus was removed and stored at -20°C (0 h p.i.). Remaining tissue culture plates were further incubated and 4h, 8h, 16h, 24h, and 48h p.i. supernatants were removed and stored at -20°C. Supernatants were centrifuged and titrated according to standard methods. The TCID$_{50}$ at the different time points after infection showed that the VP5 expressing virus (IBDV/EK) replicated faster than the virus mutant lacking VP5 (IBDV/VP5⁻). 16 h after infection IBDV/EK showed a 100-fold higher than IBDV/VP5⁻ (Fig. 5). However, at 48 h p.i. IBDV/VP5⁻ reached a titre of $10^{7.2}$ TCID$_{50}$/ml which was similar to IBDV/EK ($10^{7.45}$/ml)

[0077] **Preparation of recombinant IBDV VP5⁻-2.** Plasmid pAD78/VP5⁻-2 was prepared by techniques similar to those described above. The nucleotide sequence of part of the mutated VP5 gene is shown in SEQ ID No. 7 and Figure 3. A restriction enzyme fragment harbouring the mutations was used to substitute the wild-type EcoRI - NdeI fragment in pAD78/EK. An outline of the protocol for the preparation of the recombinant plasmid is shown in Figure 3. The organisation of pBD78 is also depicted in Figure 3. The recombinant virus was prepared as described above, except for the fact that segment B of strain D78 (SEQ ID No. 8) was used and QM-7 cells were used for the transfection experiment.

Example 2

Identification of VP5 protein in different IBDV strains

[0078] Different strains of IBDV were investigated for the expression of the VP5-gene. This was done by making use of the immuno-fluorescence technique (IFT). Chicken embryo fibroblasts grown in microtiterplates were infected with different IBDV strains. Three to 5 days after incubation at 37°C cells were fixed with 70% ethanol, then treated with polyclonal rabbit anti IBDV serum (R1928), polyclonal rabbit anti VP5 serum (RαVP5) or monoclonal antibody directed against VP5 (DIE7), respectively. Binding of the poly- or monoclonal antibodies to the different IBDV strains was visualised by making use of a fluorescence labelled conjugate (goat-anti-rabbit or goat-anti-mouse). The results are shown in Table 2:

Table 2

Identification of different sero- and subtypes of IBDV strains. Determination of the presence of VP5 proteins.					
IBDV-serotype	IBDV-subtype	IBDV-strain	R1928	RαVP5	DIE7
I	Classical	D78	+	+	+
I	Classical	228TC	+	+	+
I	Classical	PBG98	+	+	+
I	Classical	Ram0404	+	+	+
I	Classical	IBDV/EK	+	+	+
I	Classical	IBDV/VP5⁻	+	-	-

Table 2 (continued)

Identification of different sero- and subtypes of IBDV strains. Determination of the presence of VP5 proteins.					
IBDV-serotype	IBDV-subtype	IBDV-strain	R1928	RαVP5	DIE7
I	GLS	GLS	+	+	+
I	Variant-E	8903	+	+	+
II	TY89	TY89	+	+	+

[0079]   From these data it can be concluded that the different strains of IBDV belonging to different sero- and subtypes do express the VP5-gene. Furthermore, the recombinant VP5⁻ IBDV vaccine strain can be differentiated from field and vaccine viruses, thereby enabling the recombinant VP5⁻ virus to be used as a marker vaccine.

Example 3

In vivo testing of the recombinant VP5⁺ and VP5⁻ IBDV vaccines in comparison with a commercial available live IBDV vaccine.

[0080]   **Preparation of IBDV vaccine.** Primary chicken embryo fibroblast (CEF) cells were prepared at a final concentration of 2×10^6/ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 25 ml of this cell suspension 0.1 ml IBDV/EK or IBDV/VP5⁻ virus (having an infectious titre of about 3.0 log10 $TCID_{50}$/ml) was added. After incubation for 5 days in a high-humidity incubator at 37°C, the total suspension was used in the animal experiment without further purification. The infectious titre of the supernatant was $10^{7.1}$ TCID50/ml.

[0081]   **Animal experiment.** In this study the potency of different vaccines (VP5 positive strain IBDV/EK and a VP5 negative strain IBDV/VP5⁻, and the commercial available IBDV vaccine Nobilis strain D78, Intervet International B.V., NL) was investigated. SPF chicks of 3 weeks old were treated as indicated in the treatment schedule.
Treatment Schedule:

Days after vaccination	Groups			
	1	2	3	4
00	IBDV/EK	IBDV/VP5⁻	D78	-
03	x	x1	x	x
07	x,bl	x1,bl	x,b	x,bl
14	x,bl	x,bl	x,bl	x,bl
20	x,bl	x,bl	x,bl	x,bl
21	ch	ch	ch	ch
24	x	x	x	x
31	+	+	+	+

VP5⁺ Bursal disease vaccination with VP5 positive vaccine clone, eye-drop route, dose $10^{4.6}$ $TCID_{50}$/animal, 0.1 ml/animal.
VP5⁻ Bursal disease vaccination with VP5 negative vaccine clone, eye-drop route, dose $10^{5.9}$ $TCID_{50}$/animal, 0.1 ml/animal.
D78 Bursal disease vaccination with IBDV VACCINE NOBILIS STRAIN D78, eye-drop route, one field dose.
ch Challenge with Bursal disease virus, Farragher strain F52/70, eye-drop route, dose $10^{2.0}$ CID_{50}/animal, 0.1 ml/animal.
bl Serological examination; VN-test and/or Western blotting.
x Histological examination (H.E. staining) and MCA-8 ELISA on bursae.
x1 Histological examination (H.E. staining) and MCA-8 ELISA on bursae and reisolation of virus from bursa of Fabricius.
+ Clinical examination and after 10 days histological examination of the bursa.

Detection of virus in the bursa of Fabricius.

[0082] Three, 7, 14 and 20 days after eye-drop vaccination, animals were sacrificed and blood and bursae obtained. The presence of virus in the bursa was determined with an enzyme-linked immunosorbent assay (ELISA) making use of the monoclonal antibody 8 (MAB-8). MAB-8 is directed specifically against IBDV. Data are depicted in Table 3.

[0083] Furthermore, 3 and 7 days after vaccination, bursae from animals of group 2 were investigated for the presence of the recombinant VP5⁻ virus. For that purpose bursae were homogenised and cultured on chicken embryo fibroblasts. The presence of the VP5⁻ virus was determined by IFT using polyclonal rabbit sera against IBDV or VP5 or monoclonal antibodies against VP5. From 13 out of 15 bursae (87%) investigated, VP5⁻ virus could be reisolated and identified (positive for R1928 and negative for RαVP5 and DIE7). This indicates that the virus upon animal passage is still VP5⁻, indicating that the virus is stable and does not revert to VP5⁺. Furthermore, by using the different poly- and monoclonal antibodies VP5⁻ vaccine virus can be discriminated from all other vaccine and/or field IBDV viruses. Therefore, the VP5⁻ vaccine may be used as a marker vaccine.

[0084] Three days after challenge no virus could be detected in groups 1, 2 and 3 with the MCA-8 ELISA. In contrast, all animals of group 4 (non-vaccinated control group) contained challenge virus in the bursa of Fabricius, 3 days after challenge. The results show that animals vaccinated with recombinant VP5⁺ (group 1), recombinant VP5⁻ (group 2) and IBDV vaccine Nobilis D78 (group 3) were protected against severe challenge.

Table 3

Individual data for detection of virus in the bursa of Fabricius with the MCA-8 ELISA at different days after vaccination or challenge.						
	Days after vaccination→				Days after challenge	
	3	7	14	20	3	
Group↓	Virus detection by ELISA↓					Protection↓
1 VP5⁺	2/8*	1/7	0/2	0/3	0/5	100%
2 VP5⁻	0/8	0/7	0/2	0/3	0/5	100%
3 D78	1/8	6/7	0/2	0/3	0/5	100%
4 -	0/8	0/7	0/2	0/3	5/5	0%

*Number of positive bursae per total number tested.

Detection of lesions in the bursa of Fabricius.

[0085] The microscopic average lesion score induced by the different IBDV (recombinant) vaccines or the challenge virus are depicted in Table 4.

[0086] Before challenge, animals vaccinated with the recombinant VP5⁺ IBDV vaccine (group 1) or vaccinated with IBDV vaccine Nobilis D78 (group 3) showed mild to moderate lesions in the bursa. Three days after challenge only chronic lesions were observed in the bursa of Fabricius, indicating that the animals of groups 1 and 3 were protected against challenge. Furthermore, 10 days after challenge only very mild lesions (0-20% lymphocytic depletion) were observed in the bursa of the animals vaccinated with VP5⁺ recombinant IBDV vaccine or with Nobilis vaccine D78. In contrast animals not vaccinated and challenged showed severe lesions 10 days after challenge. In other words all animals (100%) of groups 1 and 3, vaccinated with the VP5⁺ recombinant IBDV vaccine or with Nobilis vaccine D78 were protected against severe challenge.

[0087] Three, 7, 14 and 20 days after vaccination and 3 and 10 days after challenge with the recombinant VP5⁻ IBDV vaccine, animals of group 2 showed no to hardly any lesions (0-20% lymphocytic depletion) in the bursa. All animals of group 2, vaccinated with the VP5⁻ recombinant IBDV vaccine, were protected against severe challenge. When animals vaccinated with the recombinant VP5⁻ IBDV vaccine are compared to animals of groups 1 or 3 (vaccinated with a recombinant VP5⁺ or commercial available vaccine) the recombinant VP5⁻ vaccine induces less lesions and therefore, is safer, milder than the vaccines tested in this experiment.

[0088] Three days post-challenge, all non-vaccinated animals of group 4 showed severe acute lesions in the bursa (total lymphocyte depletion, score 5.0). Ten days after challenge, all animals (17 out of 17 animals) showed total lymphocytic depletion, indicating that these animals were not protected against severe challenge. Animals that died after challenge, all showed severe lesions in the bursa of Fabricius. It was concluded that control group 4 was not protected against severe challenge indicating that the test conditions were optimal.

Table 4

Average bursal lesion score at different days after vaccination or challenge. The average lesion score is calculated as follows: all lesion scores from the animals per group on a certain day are added. This number is then divided by the total number of animals investigated in that group on that day. Individual scores range from 1 to 5. Score 0 = no lymphocytic depletion, score 1 = 0 - 20%; score 2 = 20 - 40%; score 3 = 40 - 60%; score 4 = 60 - 80% and score 5 = 80 - 100 % lymphocytic depletion (total lymphocytic depletion).							
	Days after vaccination→				Days after challenge→		
	3	7	14	20	3	10	
Group↓	Bursal lesions score↓						Protection↓
1 VP5$^+$	0.8	2.9	1.0	1.0	1.0^c	0.6	100%
2 VP5$^-$	0.0	0.0	0.5	0.0	0.0^c	0.1	100%
3 D78	0.1	2.4	3.5	2.0	2.8^c	1.1	100%
4 -	0.0	0.0	0.0	0.0	5.0^a	5.0	0%

^{a} Acute lesions

^{c} Chronic lesions

Serological response.

[0089] The serological response of the animals was determined by measuring the ability of blood serum to neutralise a classical infectious bursal disease virus strain in a virus neutralising (VN) test. Serum was investigated 3, 7, 14 and 20 days after vaccination. The average neutralising titres are shown in Table 5.

[0090] The results show that recombinant IBDV vaccine VP5$^+$ applied to chickens of group 1 induced a good and high serological response 20 days after vaccination which is comparable to the serological response of the chickens vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3). The recombinant IBDV vaccine VP5$^-$ applied to chickens of group 2 induced also a good serological response. A titre of 9.4 log2 was observed 20 days after vaccination. The serological response induced by the recombinant VP5$^-$ IBDV vaccine was delayed when compared to the serological response induced by the recombinant IBDV VP5$^+$ vaccine or the commercial IBDV vaccine Nobilis strain D78.

[0091] The non-vaccinated group 4 showed no serological response to IBDV.

Table 5

Average IBDV-VN-titres for groups 1 to 4 at different days after vaccination, expressed as log2 of the dilution.				
Group	Days after vaccination			
	3	7	14	20
1 VP5$^+$	≤ 1.0 ± 0.0	7.1 ± 1.7	10.2 ± 1.4	11.9 ± 1.8
2 VP5$^-$	≤ 1.0 ± 0.0	2.1 ± 1.7	6.3 ± 2.9	9.4 ± 1.4
3 D78	≤ 1.0 ± 0.0	5.2 ± 2.8	10.3 ± 1.3	11.6 ± 1.5
4-	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0	≤ 1.0 ± 0.0

Serological differentiation between antisera.

[0092] The serological response against VP5 was investigated by making use of western blot analysis. For this purpose the VP5 protein was expressed in the E. coli or baculo expression system. The expressed proteins were separated by SDS PAGE. Next the proteins were electroblotted onto a nitro-cellulose membrane. Thereafter, the membrane was cut into lanes and the lanes were incubated with rabbit anti-VP5 serum, chicken serum directed against VP5$^+$ recombinant vaccine, chicken serum directed against VP5$^-$ recombinant vaccine or negative serum from SPF chickens. Data are summarised in Table 6. As can be seen from Table 6, the VP5$^-$ serum does not induce a serological response against VP5. In contrast the rabbit anti-VP5 serum and chicken serum directed against VP5$^+$ recombinant vaccine do recognise the VP5-protein and thus induces a serological response against VP5. This indicates that chicken serum may be used to investigate if animals are exposed to a virus that expresses the VP5 protein (e.g. field virus) or

to the VP5⁻ recombinant vaccine.

Table 6

Western blot analysis. Serum from animals vaccinated with VP5⁺ or VP5⁻recombinant vaccine as well as SPF chicken serum and anti VP5-rabbit serum were investigated for their reaction with the VP5-protein.	
Identification of serum sample	Immuno-blot
VP5⁺ vaccinated animal, serum sample 20d after vaccination	positive
VP5⁻ vaccinated animal, serum sample 20d after vaccination	negative
Non-vaccinated control, serum sample at 20d	negative
Rabbit anti VP5 serum	positive

Mortality and clinical signs.

[0093] None of the animals vaccinated with VP5⁺ IBDV vaccine (group 1), vaccinated with recombinant VP5⁻ IBDV vaccine (group 2) or vaccinated with the commercial IBDV vaccine Nobilis strain D78 (group 3), died or showed clinical signs of infectious bursal disease after challenge, indicating that the animals were protected against severe challenge. All animals in the non-vaccinated control group were not protected against severe challenge.

Example 4

In vivo testing of the recombinant VP5⁻-2 vaccine

[0094] **Preparation of the IBDV vaccines.** Primary chicken embryo fibroblasts (CEF) cells were prepared at a final concentration of 2×10^6/ml. The cells were cultured in Eagles minimum essential medium containing 5% fetal calf serum. To 15 ml of this cell suspension 0.1 ml IBDV/VP5⁻-2 (D78/D78/VP5⁻) virus was added. After incubation for 6 days in a high humidity incubator at 37°C, the supernatant was titrated. The infectious titre of the supernatant was $10^{8.2}$ TCID$_{50}$/ml. For the second animal experiment the supernatant was diluted to result in a vaccine dose of $10^{5.5}$ TCID$_{50}$/animal and for the first animal experiment the supernatant was diluted to result in a vaccine dose of $10^{4.0}$ TCID$_{50}$/animal or $10^{5.0}$ TCID$_{50}$/egg.

[0095] **First animal experiment.** The effect of the vaccine is assessed by measurement of the serological response and resistance to challenge obtained from administering a challenge virus at the age of 14 days. The vaccine ($10^{5.0}$ TCID$_{50}$/egg or $10^{4.0}$ TCID$_{50}$/animal of D78/D78/VP5⁻) was applied *in ovo* or intramuscularly at day old. Microscopic lesions in the bursa were investigated, 3 and 10 days after challenge. Protection against challenge was determined and the serological response at the age of 14 days old was determined with the VN-test.

1.   Average microscopic lesion score in the bursa 3 and 10 days after challenge.

Days post	Group		
challenge	*In ovo*	Day old	None-vaccinated
3	3.3	0.0	5.0
10	0.2	0.0	5.0

2.   Protection after challenge

	Group		
	In ovo	Day old	None-vaccinated
% protection	91.6	100	0

3. Serological response against IBDV

	Group		
	In ovo	Day old	None-vaccinated
VN-titre	6.4 ± 1.7	6.4 ± 1.3	<4.0 ± 0.0

VN-titre is expressed as log2 of the dilution. Animals with a titre <4.0 log2 are considered negative

Conclusions

[0096]

1   The D78/D78/VP5⁻ strain is a highly attenuated IBD-virus
2   The virus strain is very mild
3   The virus can induce a serological response
4   The virus can induce protection
5   The virus strain can be applied by intramuscular injection to 1 day old SPF chickens and *in ovo* to 18-days-old embryonated SPF-eggs

[0097]   **Second animal experiment.** The effect of the vaccine is assessed by measurement of the serological response against IBDV and resistance to challenge obtained from administering a challenge virus, 21 days after administering the Gumboro vaccine. The vaccine ($10^{5.5}$ $TCID_{50}$/animal of D78/D78/VP5⁻) was applied via the intramuscular route to 14 days old SPF-chickens. Three, 7, 14, and 20 days after vaccination and 3 days after challenge Bursa, spleen, thymus, liver, duodenum, pancreas, ceacal tonsils and harderian gland were investigated for microscopic lesions. Ten days after challenge Bursae were investigated for microscopic lesions. Sera were tested in the VN-test. And mortality was scored after challenge.

1. Percentage mortality after challenge:	
	Mortality after challenge
Vaccinated group	0%
Control group	50%

2. Microscopic lesions of the vaccinated group before and after challenge:							
Days post Vaccinat.	Bursa	Spleen	Thymus	Liver	Duodeum	Pancreas	Ceacal Tonsils	Harderian Gland
3	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0

(continued)

2. Microscopic lesions of the vaccinated group before and after challenge:								
Days post Vaccinat.	Bursa	Spleen	Thymus	Liver	Duodeum	Pancreas	Ceacal Tonsils	Harderian Gland
14	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
24	0,A	0	0	0	0	0	0	0
31	0,A	ND	ND	ND	ND	ND	ND	ND
A = None vaccinated animals showed a lymphocytic depletion score of 5.0 (100%) and 4.25, 3 and 10 days after challenge, respectively. ND = not done.								

3. Serological response after vaccination:
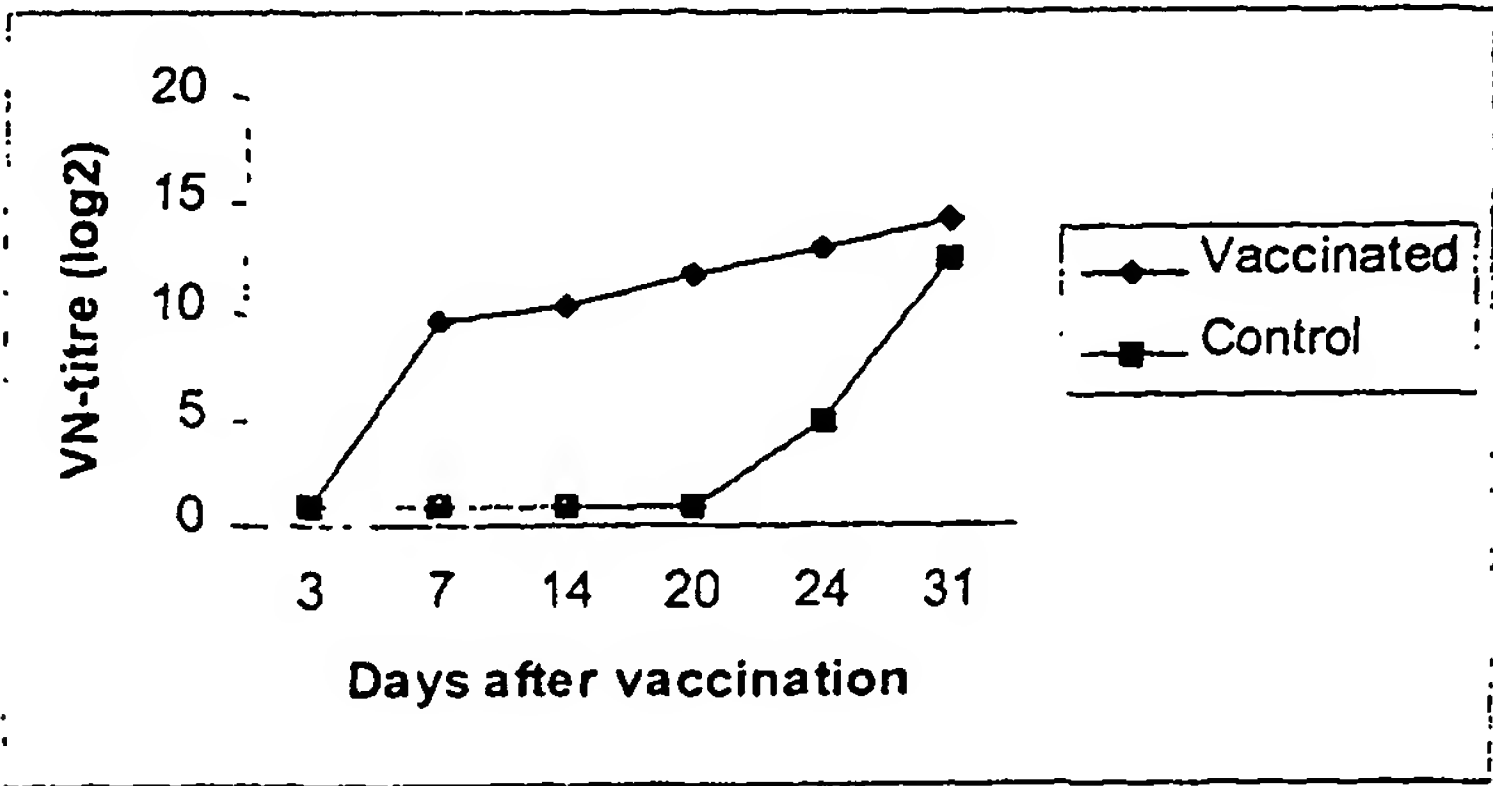
Conclusions

[0098]

1. The D78/D78/VP5⁻ strain is a highly attenuated IBD-virus
2. The virus strain is very mild and does not induce lesions in organs
3. The virus can induce a serological response
4. The virus can induce protection

LEGENDS TO THE FIGURES

[0099] Figure 1 Genomic organization of segment A and segment B of IBDV. The numbers indicate the nucleotide positions of the start, end and coding region on the segments.

[0100] Figure 2 Construction of genomic cDNA clones for the preparation of IBDV/VP5⁻. Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBP2 contains the complete strain P2 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5⁻ altering the methionine start codon for VP5 into arginine and creating an artificial Afl II cleavage site. Recombinant plasmids were linearized with the underlined restriction enzymes, followed by T7 polymerase transcription.

[0101] Figure 3 Construction of genomic cDNA clones for the preparation of IBDV/VP5⁻-2. Plasmid pAD78/EK contains the complete D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBD78 contains the complete strain D78 segment B encoding VP1. Mutations were introduced in plasmid pAD78/VP5⁻ altering the methionine start codon for VP5 into glutamic acid and creating an artificial BstBI cleavage site. Further mutations were introduced in the arginine and glutamine codon. Recombinant plasmids were linearized with the underlined restriction

enzymes, followed by T7 polymerase transcription.

[0102] Figure 4 Radioimmunoprecipitation of proteins from CEC infected cells with recombinant IBDV. CEC infected cells with IBDV/VP5⁻ (lanes 1-3), IBDV/EK (lanes 4-6) and uninfected controls were immunoprecipitated with rabbit anti-IBDV serum (lanes 1, 4, 7), rabbit anti-VP5 serum (lanes 2, 5, 8) and mAb DIE 7 (lanes 3, 6, 9). Position of molecular mass markers (M) is indicated. Location of the viral proteins VP2, VP3, VP4 and VP5 are marked.

[0103] Figure 5 Replication kinetics of IBDV/EK and IBDV/VP5⁻. Infectious titers of supernatants (vertical axis) are determined at the times indicated.

SEQUENCE LISTING

[0104]

(1) GENERAL INFORMATION:

   (i) APPLICANT:

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   (ii) TITLE OF INVENTION: Recombinant birnavirus vaccine

   (iii) NUMBER OF SEQUENCES: 8

   (iv) COMPUTER READABLE FORM:

      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: Patent In Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 2827 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (ix) FEATURE:

      (A) NAME/KEY: CDS
      (B) LOCATION:112..2745

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGATACGATG GGTCTGACCC TCTGGGAGTC ACGAATTAAC GTGGCTACTA GGGGCGATAC   60

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                CCGCCGCTGG CCGCCACGTT AGTGGCTCCT CTTCTTGATG ATTCTGCCAC C ATG AGT        117
                                                                        Met Ser
                                                                          1

                GAC ATT TTC AAC AGT CCA CAG GCG CGA AGC ACG ATC TCA GCA GCG TTC           165
                Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala Ala Phe
                          5                   10                  15

                GGC ATA AAG CCT ACT GCT GGA CAA GAC GTG GAA GAA CTC TTG ATC CCT           213
                Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu Ile Pro
                        20                  25                  30

                AAA GTT TGG GTG CCA CCT GAG GAT CCG CTT GCC AGC CCT AGT CGA CTG           261
                Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser Arg Leu
                35                  40                  45                  50

                GCA AAG TTC CTC AGA GAG AAC GGC TAC AAA GTT TTG CAG CCA CGG TCT           309
                Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro Arg Ser
                                55                  60                  65

                CTG CCC GAG AAT GAG GAG TAT GAG ACC GAC CAA ATA CTC CCA GAC TTA           357
                Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro Asp Leu
                              70                  75                  80

                GCA TGG ATG CGA CAG ATA GAA GGG GCT GTT TTA AAA CCC ACT CTA TCT           405
                Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr Leu Ser
                            85                  90                  95

                CTC CCT ATT GGA GAT CAG GAG TAC TTC CCA AAG TAC TAC CCA ACA CAT           453
                Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro Thr His
                        100                 105                 110

                CGC CCT AGC AAG GAG AAG CCC AAT GCG TAC CCG CCA GAC ATC GCA CTA           501
                Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile Ala Leu
                115                 120                 125                 130

                CTC AAG CAG ATG ATT TAC CTG TTT CTC CAG GTT CCA GAG GCC AAC GAG           549
                Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala Asn Glu
                            135                 140                 145

                GGC CTA AAG GAT GAA GTA ACC CTC TTG ACC CAA AAC ATA AGG GAC AAG           597
                Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg Asp Lys
                            150                 155                 160

                GCC TAT GGA AGT GGG ACC TAC ATG GGA CAA GCA AAT CGA CTT GTG GCC           645
                Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu Val Ala
                            165                 170                 175
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ATG AAG GAG GTC GCC ACT GGA AGA AAC CCA AAC AAG GAT CCT CTA AAG        693
Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro Leu Lys
        180             185                 190

CTT GGG TAC ACT TTT GAG AGC ATC GCG CAG CTA CTT GAC ATC ACA CTA        741
Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile Thr Leu
195             200                 205                     210

CCG GTA GGC CCA CCC GGT GAG GAT GAC AAG CCC TGG GTG CCA CTC ACA        789
Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr
                215                 220                 225

AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC        837
Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp
            230                 235                 240

TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT        885
Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser
            245                 250                 255

GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG        933
Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met
            260                 265                 270

ATA GCT ATC TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG        981
Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys
275                 280                 285                 290

CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG       1029
Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu Ser Met
                295                 300                 305

TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT       1077
Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala
                310                 315                 320

GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG       1125
Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn Ile Trp
                325                 330                 335

TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCT ATG ATC ACC TGG CCC       1173
Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro
                340                 345                 350

GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA       1221
Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro
355                 360                 365                 370
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· TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC          1269
  Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg Ile Val
              375             380             385

  GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC          1317
  Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp
              390             395             400

  AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG          1365
  Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu
              405             410             415

  AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC          1413
  Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr
              420             425             430

  TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT          1461
  Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn
  435             440             445             450

  CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG          1509
  Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val
              455             460             465

  GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA          1557
  Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln
              470             475             480

  GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACA          1605
  Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr
              485             490             495

  CTA GTG CTT GAC CAG TGG AAC CTG ATG AGA CAG CCC AGA CCA GAC AGC          1653
  Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser
              500             505             510

  GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT          1701
  Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile
  515             520             525             530

  GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC          1749
  Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu
              535             540             545

  CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC          1797
  Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu Gln Ser
              550             555             560
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'AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC     1845
 Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser
         565                 570                 575

 AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT     1893
 Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe
         580                 585                 590

 TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC     1941
 Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu Lys Ser
 595                 600                 605                 610

 AAA GTC GGG ATC GAG CAG GCA TAC AAG GTA GTC AGG TAT GAG GCG TTG     1989
 Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu Ala Leu
                 615                 620                 625

 AGG TTG GTA GGT GGT TGG AAC TAC CCA CTC CTG AAC AAA GCC TGC AAG     2037
 Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala Cys Lys
                 630                 635                 640

 AAT AAC GCA GGC GCC GCT CGG CGG CAT CTG GAG GCC AAG GGG TTC CCA     2085
 Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly Phe Pro
                 645                 650                 655

 CTC GAC GAG TTC CTA GCC GAG TGG TCT GAG CTG TCA GAG TTC GGT GAG     2133
 Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe Gly Glu
                 660                 665                 670

 GCC TTC GAA GGC TTC AAT ATC AAG CTG ACC GTA ACA TCT GAG AGC CTA     2181
 Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu Ser Leu
 675                 680                 685                 690

 GCC GAA CTG AAC AAG CCA GTA CCC CCC AAG CCC CCA AAT GTC AAC AGA     2229
 Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val Asn Arg
                 695                 700                 705

 CCA GTC AAC ACT GGG GGA CTC AAG GCA GTC AGC AAC GCC CTC AAG ACC     2277
 Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu Lys Thr
                 710                 715                 720

 GGT CGG TAC AGG AAC GAA GCC GGA CTG AGT GGT CTC GTC CTT CTA GCC     2325
 Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu Leu Ala
                 725                 730                 735

 ACA GCA AGA AGC CGT CTG CAA GAT GCA GTT AAG GCC AAG GCA GAA GCC     2373
 Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala Glu Ala
                 740                 745                 750
```

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GAG AAA CTC CAC AAG TCC AAG CCA GAC GAC CCC GAT GCA GAC TGG TTC          2421
Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp Trp Phe
755             760             765             770


GAA AGA TCA GAA ACT CTG TCA GAC CTT CTG GAG AAA GCC GAC ATC GCC          2469
Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp Ile Ala
            775             780             785


AGC AAG GTC GCC CAC TCA GCA CTC GTG GAA ACA AGC GAC GCC CTT GAA          2517
Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu
            790             795             800


GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG          2565
Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys
            805             810             815


AAC CCA CAG ACC GCC TCC AAC CCC GTT GTT GGG CTC CAC CTG CCC GCC          2613
Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala
    820             825             830


AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC          2661
Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser
835             840             845             850


AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA          2709
Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys
            855             860             865


ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT             2755
Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg
            870             875


GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT        2815


GCGGGGGCCC CC                                                          2827
```

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 878 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ser Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala
 1               5                  10                 15

Ala Phe Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu
                20                 25                 30

Ile Pro Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser
            35                 40                 45

Arg Leu Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro
         50                 55                 60

Arg Ser Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro
 65              70                 75                 80

Asp Leu Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr
                85                 90                 95

Leu Ser Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro
             100                105                110

Thr His Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile
         115                120                125

Ala Leu Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala
     130                135                140

Asn Glu Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg
145             150                155                160

Asp Lys Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Asn Arg Leu
                165                170                175

Val Ala Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro
             180                185                190

Leu Lys Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile
         195                200                205

Thr Leu Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro
     210                215                220

Leu Thr Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp
225                230                235                240

Gly Asp Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser
                245                250                255
```

Ser Ser Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly
              260   265   270

Glu Met Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu
              275   280   285

Leu Lys Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu
              290   295   300

Ser Met Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro
305   310   315   320

Lys Ala Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn
                  325   330   335

Ile Trp Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr
              340   345   350

Trp Pro Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly
              355   360   365

Cys Pro Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg
      370   375   380

Ile Val Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr
385   390   395   400

Ala Asp Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp
              405   410   415

Leu Glu Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala
              420   425   430

Met Tyr Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met
           435   440   445

Phe Asn Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu
      450   455   460

Val Val Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr
465   470   475   480

Gly Gln Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu
              485   490   495

Ser Thr Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro
           500   505   510

Asp Ser Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe
      515   520   525

Lys Ile Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu
      530   535   540

Val Leu Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu
      545   550   555   560

Gln Ser Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr
          565   570   575

Tyr Ser Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg
         580   585   590

Leu Phe Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu
         595   600   605

Lys Ser Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu
         610   615   620

Ala Leu Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala
625   630   635   640

Cys Lys Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly
         645   650   655

Phe Pro Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe
         660   665   670

Gly Glu Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu
         675   680   685

Ser Leu Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val
         690   695   700

Asn Arg Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu
705   710   715   720

Lys Thr Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu
         725   730   735

Leu Ala Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala
         740   745   750

Glu Ala Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp
         755   760   765

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Trp Phe Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp
    770             775             780

Ile Ala Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala
785             790             795             800

Leu Glu Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu
                805             810             815

Val Lys Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu
            820             825             830

Pro Ala Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly
        835             840             845

Thr Ser Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala
    850             855             860

Val Lys Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg
865             870             875
```

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 3261 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:

        (A) NAME/KEY: CDS
        (B) LOCATION:97..531

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTC          60

CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTG ATG GTT AGT AGA GAT CAG          114
                                        Met Val Ser Arg Asp Gln
                                         1                   5
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ACA AAC GAT CGC AGC GAT GAC AAA CCT GCA AGA TCA AAC CCA ACA GAT         162
Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala Arg Ser Asn Pro Thr Asp
             10                  15                  20

TGT TCC GTT CAT ACG GAG CCT TCT GAT GCC AAC AAC CGG ACC GGC GTC         210
Cys Ser Val His Thr Glu Pro Ser Asp Ala Asn Asn Arg Thr Gly Val
         25                  30                  35

CAT TCC GGA CGA CAC CCT GGA GAA GCA CAC TCT CAG GTC AGA GAC CTC         258
His Ser Gly Arg His Pro Gly Glu Ala His Ser Gln Val Arg Asp Leu
     40                  45                  50

GAC CTA CAA TTT GAC TGT GGG GGA CAC AGG GTC AGG GCT AAT TGT CTT         306
Asp Leu Gln Phe Asp Cys Gly Gly His Arg Val Arg Ala Asn Cys Leu
 55                  60                  65                  70

TTT CCC TGG ATT CCC TGG CTC AAT TGT GGG TGC TCA CTA CAC ACT GCA         354
Phe Pro Trp Ile Pro Trp Leu Asn Cys Gly Cys Ser Leu His Thr Ala
                 75                  80                  85

GGG CAA TGG GAA CTA CAA GTT CGA TCA GAT GCT CCT GAC TGC CCA GAA         402
Gly Gln Trp Glu Leu Gln Val Arg Ser Asp Ala Pro Asp Cys Pro Glu
             90                  95                  100

CCT ACC GGC CAG TTA CAA CTA CTG CAG GCT AGT GAG TCG GAG TCT CAC         450
Pro Thr Gly Gln Leu Gln Leu Leu Gln Ala Ser Glu Ser Glu Ser His
         105                 110                 115

AGT GAG GTC AAG CAC ACT TCC TGG TGG CGT TTT TGC ACT AAA CGG CAC         498
Ser Glu Val Lys His Thr Ser Trp Trp Arg Leu Cys Thr Lys Arg His
         120                 125                 130

CAT AAA CGC CGT GAC CTT CCA AGG AAG CCT GAG TGAACTGACA GATGTTAGCT       551
His Lys Arg Arg Asp Leu Pro Arg Lys Pro Glu
135                 140                 145

ACAATGGGTT GATGTCTGCA ACAGCCAACA TCAACGACAA AATTGGGAAC GTCCTAGTAG       611

GGGAAGGGGT CACCGTCCTC AGCTTACCCA CATCATATGA TCTTGGGTAT GTGAGGCTTG       671

GTGACCCCAT TCCCGCAATA GGGCTTGACC CAAAAATGGT AGCCACATGT GACAGCAGTG       731

ACAGGCCCAG AGTCTACACC ATAACTGCAG CCGATGATTA CCAATTCTCA TCACAGTACC       791

AACCAGGTGG GGTAACAATC ACACTGTTCT CAGCCAACAT TGATGCCATC ACAAGCCTCA       851

GCGTTGGGGG AGAGCTCGTG TTTCAAACAA GCGTCCACGG CCTTGTACTG GGCGCCACCA       911
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TCTACCTCAT AGGCTTTGAT GGGACAACGG TAATCACCAG GGCTGTGGCC GCAAACAATG    971

GGCTGACGAC CGGCACCGAC AACCTTATGC CATTCAATCT TGTGATTCCA ACAAACGAGA   1031

TAACCCAGCC AATCACATCC ATCAAACTGG AGATAGTGAC CTCCAAAAGT GGTGGTCAGG   1091

CAGGGGATCA GATGTCATGG TCGGCAAGAG GGAGCCTAGC AGTGACGATC CATGGTGGCA   1151

ACTATCCAGG GGCCCTCCGT CCCGTCACGC TAGTGGCCTA CGAAAGAGTG GCAACAGGAT   1211

CCGTCGTTAC GGTCGCTGGG GTGAGCAACT TCGAGCTGAT CCCAAATCCT GAACTAGCAA   1271

AGAACCTGGT TACAGAATAC GGCCGATTTG ACCCAGGAGC CATGAACTAC ACAAAATTGA   1331

TACTGAGTGA GAGGGACCGT CTTGGCATCA AGACCGTCTG GCCAACAAGG GAGTACACTG   1391

ACTTTCGTGA ATACTTCATG GAGGTGGCCG ACCTCAACTC TCCCCTGAAG ATTGCAGGAG   1451

CATTCGGCTT CAAAGACATA ATCCGGGCCA TAAGGAGGAT AGCTGTGCCG GTGGTCTCCA   1511

CATTGTTCCC ACCTGCCGCT CCCCTAGCCC ATGCAATTGG GGAAGGTGTA GACTACCTGC   1571

TGGGCGATGA GGCACAGGCT GCTTCAGGAA CTGCTCGAGC CGCGTCAGGA AAAGCAAGAG   1631

CTGCCTCAGG CCGCATAAGG CAGCTGACTC TCGCCGCCGA CAAGGGGTAC GAGGTAGTCG   1691

CGAATCTATT CCAGGTGCCC CAGAATCCCG TAGTCGACGG GATTCTTGCT TCACCTGGGG   1751

TACTCCGCGG TGCACACAAC CTCGACTGCG TGTTAGAGA GGGTGCCACG CTATTCCCTG   1811

TGGTTATTAC GACAGTGGAA GACGCCATGA CACCCAAAGC ATTGAACAGC AAAATGTTTG   1871

CTGTCATTGA AGGCGTGCGA GAAGACCTCC AACCTCCATC TCAAAGAGGA TCCTTCATAC   1931

GAACTCTCTC TGGACACAGA GTCTATGGAT ATGCTCCAGA TGGGGTACTT CCACTGGAGA   1991

CTGGGAGAGA CTACACCGTT GTCCCAATAG ATGATGTCTG GGACGACAGC ATTATGCTGT   2051

CCAAAGATCC CATACCTCCT ATTGTGGGAA ACAGTGGAAA TCTAGCCATA GCTTACATGG   2111

ATGTGTTTCG ACCCAAAGTC CCAATCCATG TGGCTATGAC GGGAGCCCTC AATGCTTGTG   2171

GCGAGATTGA GAAAGTAAGC TTTAGAAGCA CCAAGCTCGC CACTGCACAC CGACTTGGCC   2231

TTAGGTTGGC TGGTCCCGGA GCATTCGATG TAAACACCGG GCCCAACTGG GCAACGTTCA   2291

TCAAACGTTT CCCTCACAAT CCACGCGACT GGGACAGGCT CCCCTACCTC AACCTACCAT   2351
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ACCTTCCACC CAATGCAGGA CGCCAGTACC ACCTTGCCAT GGCTGCATCA GAGTTCAAAG        2411

AGACCCCCGA ACTCGAGAGT GCCGTCAGAG CAATGGAAGC AGCAGCCAAC GTGGACCCAC        2471

TATTCCAATC TGCACTCAGT GTGTTCATGT GGCTGGAAGA GAATGGGATT GTGACTGACA        2531

TGGCCAACTT CGCACTCAGC GACCCGAACG CCCATCGGAT GCGAAATTTT CTTGCAAACG        2591

CACCACAAGC AGGCAGCAAG TCGCAAAGGG CCAAGTACGG GACAGCAGGC TACGGAGTGG        2651

AGGCTCGGGG CCCCACACCA GAGGAAGCAC AGAGGGAAAA AGACACACGG ATCTCAAAGA        2711

AGATGGAGAC CATGGGCATC TACTTTGCAA CACCAGAATG GGTAGCACTC AATGGGCACC        2771

GAGGGCCAAG CCCCGGCCAG CTAAAGTACT GGCAGAACAC ACGAGAAATA CCGGACCCAA        2831

ACGAGGACTA TCTAGACTAC GTGCATGCAG AGAAGAGCCG GTTGGCATCA GAAGAACAAA        2891

TCCTAAGGGC AGCTACGTCG ATCTACGGGG CTCCAGGACA GGCAGAGCCA CCCCAAGCTT        2951

TCATAGACGA AGTTGCCAAA GTCTATGAAA TCAACCATGG ACGTGGCCCA AACCAAGAAC        3011

AGATGAAAGA TCTGCTCTTG ACTGCGATGG AGATGAAGCA TCGCAATCCC AGGCGGGCTC        3071

TACCAAAGCC CAAGCCAAAA CCCAATGCTC CAACACAGAG ACCCCCTGGT CGGCTGGGCC        3131

GCTGGATCAG GACCGTCTCT GATGAGGACC TTGAGTGAGG CTCCTGGGAG TCTCCCGACA        3191

CCACCCGCGC AGGTGTGGAC ACCAATTCGG CCTTACAACA TCCCAAATTG GATCCGTTCG        3251

CGGGTCCCCT                                                              3261
```

(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 145 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Val Ser Arg Asp Gln Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala
 1               5                  10                 15
```

```
Arg Ser Asn Pro Thr Asp Cys Ser Val His Thr Glu Pro Ser Asp Ala
            20          25              30

Asn Asn Arg Thr Gly Val His Ser Gly Arg His Pro Gly Glu Ala His
        35              40              45

Ser Gln Val Arg Asp Leu Asp Leu Gln Phe Asp Cys Gly Gly His Arg
    50              55              60

Val Arg Ala Asn Cys Leu Phe Pro Trp Ile Pro Trp Leu Asn Cys Gly
65              70              75              80

Cys Ser Leu His Thr Ala Gly Gln Trp Glu Leu Gln Val Arg Ser Asp
            85              90              95

Ala Pro Asp Cys Pro Glu Pro Thr Gly Gln Leu Gln Leu Leu Gln Ala
            100             105             110

Ser Glu Ser Glu Ser His Ser Glu Val Lys His Thr Ser Trp Trp Arg
        115             120             125

Leu Cys Thr Lys Arg His His Lys Arg Arg Asp Leu Pro Arg Lys Pro
    130             135             140

Glu
145
```

(2) INFORMATION FOR SEQ ID NO: 5:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 3261 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: cDNA

   (ix) FEATURE:

      (A) NAME/KEY: CDS
      (B) LOCATION:131..3166

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTC        60
```

```
CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTGATGG TTAGTAGAGA TCAGACAAAC        120
```

```
GATCGCAGCG ATG ACA AAC CTG CAA GAT CAA ACC CAA CAG ATT GTT CCG          169
           Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro
            1               5                  10
```

```
TTC ATA CGG AGC CTT CTG ATG CCA ACA ACC GGA CCG GCG TCC ATT CCG         217
Phe Ile Arg Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro
            15              20              25
```

```
GAC GAC ACC CTG GAG AAG CAC ACT CTC AGG TCA GAG ACC TCG ACC TAC         265
Asp Asp Thr Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr
30              35              40              45
```

```
AAT TTG ACT GTG GGG GAC ACA GGG TCA GGG CTA ATT GTC TTT TTC CCT         313
Asn Leu Thr Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro
                50              55              60
```

```
GGA TTC CCT GGC TCA ATT GTG GGT GCT CAC TAC ACA CTG CAG GGC AAT         361
Gly Phe Pro Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn
                65              70              75
```

```
GGG AAC TAC AAG TTC GAT CAG ATG CTC CTG ACT GCC CAG AAC CTA CCG         409
Gly Asn Tyr Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro
                80              85              90
```

```
GCC AGT TAC AAC TAC TGC AGG CTA GTG AGT CGG AGT CTC ACA GTG AGG         457
Ala Ser Tyr Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg
        95              100             105
```

```
TCA AGC ACA CTT CCT GGT GGC GTT TAT GCA CTA AAC GGC ACC ATA AAC         505
Ser Ser Thr Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn
110             115             120             125
```

```
GCC GTG ACC TTC CAA GGA AGC CTG AGT GAA CTG ACA GAT GTT AGC TAC         553
Ala Val Thr Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr
                130             135             140
```

```
AAT GGG TTG ATG TCT GCA ACA GCC AAC ATC AAC GAC AAA ATT GGG AAC         601
Asn Gly Leu Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn
                145             150             155
```

```
GTC CTA GTA GGG GAA GGG GTC ACC GTC CTC AGC TTA CCC ACA TCA TAT         649
Val Leu Val Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr
        160             165             170
```

```
GAT CTT GGG TAT GTG AGG CTT GGT GAC CCC ATT CCC GCA ATA GGG CTT         697
```

Asp Leu Gly Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu
175 180 185

5

GAC CCA AAA ATG GTA GCC ACA TGT GAC AGC AGT GAC AGG CCC AGA GTC   745
Asp Pro Lys Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val
190 195 200 205

10

TAC ACC ATA ACT GCA GCC GAT GAT TAC CAA TTC TCA TCA CAG TAC CAA   793
Tyr Thr Ile Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln
210 215 220

15

CCA GGT GGG GTA ACA ATC ACA CTG TTC TCA GCC AAC ATT GAT GCC ATC   841
Pro Gly Gly Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile
225 230 235

20

ACA AGC CTC AGC GTT GGG GGA GAG CTC GTG TTT CAA ACA AGC GTC CAC   889
Thr Ser Leu Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His
240 245 250

GGC CTT GTA CTG GGC GCC ACC ATC TAC CTC ATA GGC TTT GAT GGG ACA   937
Gly Leu Val Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr

25

255 260 265

ACG GTA ATC ACC AGG GCT GTG GCC GCA AAC AAT GGG CTG ACG ACC GGC   985
Thr Val Ile Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly

30

270 275 280 285

ACC GAC AAC CTT ATG CCA TTC AAT CTT GTG ATT CCA ACA AAC GAG ATA   1033
Thr Asp Asn Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile

35

290 295 300

ACC CAG CCA ATC ACA TCC ATC AAA CTG GAG ATA GTG ACC TCC AAA AGT   1081
Thr Gln Pro Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser
305 310 315

40

GGT GGT CAG GCA GGG GAT CAG ATG TCA TGG TCG GCA AGA GGG AGC CTA   1129
Gly Gly Gln Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu
320 325 330

45

GCA GTG ACG ATC CAT GGT GGC AAC TAT CCA GGG GCC CTC CGT CCC GTC   1177
Ala Val Thr Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val
335 340 345

50

ACG CTA GTG GCC TAC GAA AGA GTG GCA ACA GGA TCC GTC GTT ACG GTC   1225
Thr Leu Val Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val
350 355 360 365

55

GCT GGG GTG AGC AAC TTC GAG CTG ATC CCA AAT CCT GAA CTA GCA AAG   1273

```
        Ala Gly Val Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys
                        370               375               380

  5     AAC CTG GTT ACA GAA TAC GGC CGA TTT GAC CCA GGA GCC ATG AAC TAC     1321
        Asn Leu Val Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr
                        385               390               395

 10     ACA AAA TTG ATA CTG AGT GAG AGG GAC CGT CTT GGC ATC AAG ACC GTC     1369
        Thr Lys Leu Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val
                        400               405               410

 15     TGG CCA ACA AGG GAG TAC ACT GAC TTT CGT GAA TAC TTC ATG GAG GTG     1417
        Trp Pro Thr Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val
                415               420               425

 20     GCC GAC CTC AAC TCT CCC CTG AAG ATT GCA GGA GCA TTC GGC TTC AAA     1465
        Ala Asp Leu Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys
        430                 435               440                 445

 25     GAC ATA ATC CGG GCC ATA AGG AGG ATA GCT GTG CCG GTG GTC TCC ACA     1513
        Asp Ile Ile Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr
                        450               455               460

 30     TTG TTC CCA CCT GCC GCT CCC CTA GCC CAT GCA ATT GGG GAA GGT GTA     1561
        Leu Phe Pro Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val
                        465               470               475

 35     GAC TAC CTG CTG GGC GAT GAG GCA CAG GCT GCT TCA GGA ACT GCT CGA     1609
        Asp Tyr Leu Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg
                        480               485               490

        GCC GCG TCA GGA AAA GCA AGA GCT GCC TCA GGC CGC ATA AGG CAG CTG     1657
        Ala Ala Ser Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu
                        495               500               505

 40     ACT CTC GCC GCC GAC AAG GGG TAC GAG GTA GTC GCG AAT CTA TTC CAG     1705
        Thr Leu Ala Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln
        510                 515               520                 525

 45     GTG CCC CAG AAT CCC GTA GTC GAC GGG ATT CTT GCT TCA CCT GGG GTA     1753
        Val Pro Gln Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val
                        530               535               540

 50     CTC CGC GGT GCA CAC AAC CTC GAC TGC GTG TTA AGA GAG GGT GCC ACG     1801
        Leu Arg Gly Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr
                        545               550               555

 55     CTA TTC CCT GTG GTT ATT ACG ACA GTG GAA GAC GCC ATG ACA CCC AAA     1849
```

```
          Leu Phe Pro Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys
                  560             565             570

5         GCA TTG AAC AGC AAA ATG TTT GCT GTC ATT GAA GGC GTG CGA GAA GAC    1897
          Ala Leu Asn Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp
                  575             580             585

10        CTC CAA CCT CCA TCT CAA AGA GGA TCC TTC ATA CGA ACT CTC TCT GGA    1945
          Leu Gln Pro Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly
          590             595             600             605

15        CAC AGA GTC TAT GGA TAT GCT CCA GAT GGG GTA CTT CCA CTG GAG ACT    1993
          His Arg Val Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr
                          610             615             620

20        GGG AGA GAC TAC ACC GTT GTC CCA ATA GAT GAT GTC TGG GAC GAC AGC    2041
          Gly Arg Asp Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser
                          625             630             635

25        ATT ATG CTG TCC AAA GAT CCC ATA CCT CCT ATT GTG GGA AAC AGT GGA    2089
          Ile Met Leu Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly
                  640             645             650

30        AAT CTA GCC ATA GCT TAC ATG GAT GTG TTT CGA CCC AAA GTC CCA ATC    2137
          Asn Leu Ala Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile
                  655             660             665

35        CAT GTG GCT ATG ACG GGA GCC CTC AAT GCT TGT GGC GAG ATT GAG AAA    2185
          His Val Ala Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys
          670             675             680             685

40        GTA AGC TTT AGA AGC ACC AAG CTC GCC ACT GCA CAC CGA CTT GGC CTT    2233
          Val Ser Phe Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu
                          690             695             700

45        AGG TTG GCT GGT CCC GGA GCA TTC GAT GTA AAC ACC GGG CCC AAC TGG    2281
          Arg Leu Ala Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp
                          705             710             715

          GCA ACG TTC ATC AAA CGT TTC CCT CAC AAT CCA CGC GAC TGG GAC AGG    2329
          Ala Thr Phe Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg
                          720             725             730

50        CTC CCC TAC CTC AAC CTA CCA TAC CTT CCA CCC AAT GCA GGA CGC CAG    2377
          Leu Pro Tyr Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln
                  735             740             745

55        TAC CAC CTT GCC ATG GCT GCA TCA GAG TTC AAA GAG ACC CCC GAA CTC    2425
```

Tyr	His	Leu	Ala	Met	Ala	Ala	Ser	Glu	Phe	Lys	Glu	Thr	Pro	Glu	Leu
750				755					760					765

5

GAG	AGT	GCC	GTC	AGA	GCA	ATG	GAA	GCA	GCA	GCC	AAC	GTG	GAC	CCA	CTA	2473
Glu	Ser	Ala	Val	Arg	Ala	Met	Glu	Ala	Ala	Ala	Asn	Val	Asp	Pro	Leu
				770				775					780

10

TTC	CAA	TCT	GCA	CTC	AGT	GTG	TTC	ATG	TGG	CTG	GAA	GAG	AAT	GGG	ATT	2521
Phe	Gln	Ser	Ala	Leu	Ser	Val	Phe	Met	Trp	Leu	Glu	Glu	Asn	Gly	Ile
			785				790				795

15

GTG	ACT	GAC	ATG	GCC	AAC	TTC	GCA	CTC	AGC	GAC	CCG	AAC	GCC	CAT	CGG	2569
Val	Thr	Asp	Met	Ala	Asn	Phe	Ala	Leu	Ser	Asp	Pro	Asn	Ala	His	Arg
		800				805				810

20

ATG	CGA	AAT	TTT	CTT	GCA	AAC	GCA	CCA	CAA	GCA	GGC	AGC	AAG	TCG	CAA	2617
Met	Arg	Asn	Phe	Leu	Ala	Asn	Ala	Pro	Gln	Ala	Gly	Ser	Lys	Ser	Gln
	815				820				825

25

AGG	GCC	AAG	TAC	GGG	ACA	GCA	GGC	TAC	GGA	GTG	GAG	GCT	CGG	GGC	CCC	2665
Arg	Ala	Lys	Tyr	Gly	Thr	Ala	Gly	Tyr	Gly	Val	Glu	Ala	Arg	Gly	Pro
830				835				840				845

30

ACA	CCA	GAG	GAA	GCA	CAG	AGG	GAA	AAA	GAC	ACA	CGG	ATC	TCA	AAG	AAG	2713
Thr	Pro	Glu	Glu	Ala	Gln	Arg	Glu	Lys	Asp	Thr	Arg	Ile	Ser	Lys	Lys
				850				855				860

35

ATG	GAG	ACC	ATG	GGC	ATC	TAC	TTT	GCA	ACA	CCA	GAA	TGG	GTA	GCA	CTC	2761
Met	Glu	Thr	Met	Gly	Ile	Tyr	Phe	Ala	Thr	Pro	Glu	Trp	Val	Ala	Leu
			865				870				875

AAT	GGG	CAC	CGA	GGG	CCA	AGC	CCC	GGC	CAG	CTA	AAG	TAC	TGG	CAG	AAC	2809
Asn	Gly	His	Arg	Gly	Pro	Ser	Pro	Gly	Gln	Leu	Lys	Tyr	Trp	Gln	Asn
		880				885				890

40

ACA	CGA	GAA	ATA	CCG	GAC	CCA	AAC	GAG	GAC	TAT	CTA	GAC	TAC	GTG	CAT	2857
Thr	Arg	Glu	Ile	Pro	Asp	Pro	Asn	Glu	Asp	Tyr	Leu	Asp	Tyr	Val	His
	895				900				905

45

GCA	GAG	AAG	AGC	CGG	TTG	GCA	TCA	GAA	GAA	CAA	ATC	CTA	AGG	GCA	GCT	2905
Ala	Glu	Lys	Ser	Arg	Leu	Ala	Ser	Glu	Glu	Gln	Ile	Leu	Arg	Ala	Ala
910				915				920				925

50

ACG	TCG	ATC	TAC	GGG	GCT	CCA	GGA	CAG	GCA	GAG	CCA	CCC	CAA	GCT	TTC	2953
Thr	Ser	Ile	Tyr	Gly	Ala	Pro	Gly	Gln	Ala	Glu	Pro	Pro	Gln	Ala	Phe
			930				935				940

ATA	GAC	GAA	GTT	GCC	AAA	GTC	TAT	GAA	ATC	AAC	CAT	GGA	CGT	GGC	CCA	3001

55

```
     Ile Asp Glu Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro
             945                 950                 955

     AAC CAA GAA CAG ATG AAA GAT CTG CTC TTG ACT GCG ATG GAG ATG AAG        3049
     Asn Gln Glu Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys
             960                 965                 970

     CAT CGC AAT CCC AGG CGG GCT CTA CCA AAG CCC AAG CCA AAA CCC AAT        3097
     His Arg Asn Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn
             975                 980                 985

     GCT CCA ACA CAG AGA CCC CCT GGT CGG CTG GGC CGC TGG ATC AGG ACC        3145
     Ala Pro Thr Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr
     990                 995             1000                1005

     GTC TCT GAT GAG GAC CTT GAG TGAGGCTCCT GGGAGTCTCC CGACACCACC           3196
     Val Ser Asp Glu Asp Leu Glu
                     1010

     CGCGCAGGTG TGGACACCAA TTCGGCCTTA CAACATCCCA AATTGGATCC GTTCGCGGGT      3256

     CCCCT                                                                  3261
```

(2) INFORMATION FOR SEQ ID NO: 6:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 1012 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
     Met Thr Asn Leu Gln Asp Gln Thr Gln Gln Ile Val Pro Phe Ile Arg
     1               5                   10                  15

     Ser Leu Leu Met Pro Thr Thr Gly Pro Ala Ser Ile Pro Asp Asp Thr
                     20                  25                  30

     Leu Glu Lys His Thr Leu Arg Ser Glu Thr Ser Thr Tyr Asn Leu Thr
                     35                  40                  45

     Val Gly Asp Thr Gly Ser Gly Leu Ile Val Phe Phe Pro Gly Phe Pro
             50                  55                  60

     Gly Ser Ile Val Gly Ala His Tyr Thr Leu Gln Gly Asn Gly Asn Tyr
```

	65				70				75				80

```
          Lys Phe Asp Gln Met Leu Leu Thr Ala Gln Asn Leu Pro Ala Ser Tyr
5                             85              90                  95

          Asn Tyr Cys Arg Leu Val Ser Arg Ser Leu Thr Val Arg Ser Ser Thr
                        100             105                 110
10
          Leu Pro Gly Gly Val Tyr Ala Leu Asn Gly Thr Ile Asn Ala Val Thr
                        115             120                 125

          Phe Gln Gly Ser Leu Ser Glu Leu Thr Asp Val Ser Tyr Asn Gly Leu
15                      130             135                 140

          Met Ser Ala Thr Ala Asn Ile Asn Asp Lys Ile Gly Asn Val Leu Val
          145                 150             155                 160
20
          Gly Glu Gly Val Thr Val Leu Ser Leu Pro Thr Ser Tyr Asp Leu Gly
                            165             170                 175

          Tyr Val Arg Leu Gly Asp Pro Ile Pro Ala Ile Gly Leu Asp Pro Lys
25                      180             185                 190

          Met Val Ala Thr Cys Asp Ser Ser Asp Arg Pro Arg Val Tyr Thr Ile
                        195             200                 205
30
          Thr Ala Ala Asp Asp Tyr Gln Phe Ser Ser Gln Tyr Gln Pro Gly Gly
                    210             215                 220

          Val Thr Ile Thr Leu Phe Ser Ala Asn Ile Asp Ala Ile Thr Ser Leu
35        225                 230             235                 240

          Ser Val Gly Gly Glu Leu Val Phe Gln Thr Ser Val His Gly Leu Val
                            245             250                 255
40
          Leu Gly Ala Thr Ile Tyr Leu Ile Gly Phe Asp Gly Thr Thr Val Ile
                        260             265                 270

          Thr Arg Ala Val Ala Ala Asn Asn Gly Leu Thr Thr Gly Thr Asp Asn
45                      275             280                 285

          Leu Met Pro Phe Asn Leu Val Ile Pro Thr Asn Glu Ile Thr Gln Pro
                    290             295                 300
50
          Ile Thr Ser Ile Lys Leu Glu Ile Val Thr Ser Lys Ser Gly Gly Gln
          305                 310             315                 320

55        Ala Gly Asp Gln Met Ser Trp Ser Ala Arg Gly Ser Leu Ala Val Thr
```

325   330   335

Ile His Gly Gly Asn Tyr Pro Gly Ala Leu Arg Pro Val Thr Leu Val
        340   345   350

Ala Tyr Glu Arg Val Ala Thr Gly Ser Val Val Thr Val Ala Gly Val
        355   360   365

Ser Asn Phe Glu Leu Ile Pro Asn Pro Glu Leu Ala Lys Asn Leu Val
        370   375   380

Thr Glu Tyr Gly Arg Phe Asp Pro Gly Ala Met Asn Tyr Thr Lys Leu
385   390   395   400

Ile Leu Ser Glu Arg Asp Arg Leu Gly Ile Lys Thr Val Trp Pro Thr
          405   410   415

Arg Glu Tyr Thr Asp Phe Arg Glu Tyr Phe Met Glu Val Ala Asp Leu
        420   425   430

Asn Ser Pro Leu Lys Ile Ala Gly Ala Phe Gly Phe Lys Asp Ile Ile
        435   440   445

Arg Ala Ile Arg Arg Ile Ala Val Pro Val Val Ser Thr Leu Phe Pro
        450   455   460

Pro Ala Ala Pro Leu Ala His Ala Ile Gly Glu Gly Val Asp Tyr Leu
465   470   475   480

Leu Gly Asp Glu Ala Gln Ala Ala Ser Gly Thr Ala Arg Ala Ala Ser
        485   490   495

Gly Lys Ala Arg Ala Ala Ser Gly Arg Ile Arg Gln Leu Thr Leu Ala
        500   505   510

Ala Asp Lys Gly Tyr Glu Val Val Ala Asn Leu Phe Gln Val Pro Gln
        515   520   525

Asn Pro Val Val Asp Gly Ile Leu Ala Ser Pro Gly Val Leu Arg Gly
        530   535   540

Ala His Asn Leu Asp Cys Val Leu Arg Glu Gly Ala Thr Leu Phe Pro
545   550   555   560

Val Val Ile Thr Thr Val Glu Asp Ala Met Thr Pro Lys Ala Leu Asn
        565   570   575

Ser Lys Met Phe Ala Val Ile Glu Gly Val Arg Glu Asp Leu Gln Pro

		580					585					590		

Pro Ser Gln Arg Gly Ser Phe Ile Arg Thr Leu Ser Gly His Arg Val
      595   600   605

Tyr Gly Tyr Ala Pro Asp Gly Val Leu Pro Leu Glu Thr Gly Arg Asp
      610   615   620

Tyr Thr Val Val Pro Ile Asp Asp Val Trp Asp Asp Ser Ile Met Leu
625   630   635   640

Ser Lys Asp Pro Ile Pro Pro Ile Val Gly Asn Ser Gly Asn Leu Ala
                  645   650   655

Ile Ala Tyr Met Asp Val Phe Arg Pro Lys Val Pro Ile His Val Ala
              660   665   670

Met Thr Gly Ala Leu Asn Ala Cys Gly Glu Ile Glu Lys Val Ser Phe
              675   680   685

Arg Ser Thr Lys Leu Ala Thr Ala His Arg Leu Gly Leu Arg Leu Ala
      690   695   700

Gly Pro Gly Ala Phe Asp Val Asn Thr Gly Pro Asn Trp Ala Thr Phe
705   710   715   720

Ile Lys Arg Phe Pro His Asn Pro Arg Asp Trp Asp Arg Leu Pro Tyr
                  725   730   735

Leu Asn Leu Pro Tyr Leu Pro Pro Asn Ala Gly Arg Gln Tyr His Leu
                  740   745   750

Ala Met Ala Ala Ser Glu Phe Lys Glu Thr Pro Glu Leu Glu Ser Ala
              755   760   765

Val Arg Ala Met Glu Ala Ala Ala Asn Val Asp Pro Leu Phe Gln Ser
      770   775   780

Ala Leu Ser Val Phe Met Trp Leu Glu Glu Asn Gly Ile Val Thr Asp
785   790   795   800

Met Ala Asn Phe Ala Leu Ser Asp Pro Asn Ala His Arg Met Arg Asn
                  805   810   815

Phe Leu Ala Asn Ala Pro Gln Ala Gly Ser Lys Ser Gln Arg Ala Lys
                  820   825   830

Tyr Gly Thr Ala Gly Tyr Gly Val Glu Ala Arg Gly Pro Thr Pro Glu

                    835   840   845

Glu Ala Gln Arg Glu Lys Asp Thr Arg Ile Ser Lys Lys Met Glu Thr
      850   855   860

Met Gly Ile Tyr Phe Ala Thr Pro Glu Trp Val Ala Leu Asn Gly His
865   870   875   880

Arg Gly Pro Ser Pro Gly Gln Leu Lys Tyr Trp Gln Asn Thr Arg Glu
                  885   890   895

Ile Pro Asp Pro Asn Glu Asp Tyr Leu Asp Tyr Val His Ala Glu Lys
                  900   905   910

Ser Arg Leu Ala Ser Glu Glu Gln Ile Leu Arg Ala Ala Thr Ser Ile
              915   920   925

Tyr Gly Ala Pro Gly Gln Ala Glu Pro Pro Gln Ala Phe Ile Asp Glu
      930   935   940

Val Ala Lys Val Tyr Glu Ile Asn His Gly Arg Gly Pro Asn Gln Glu
945   950   955   960

Gln Met Lys Asp Leu Leu Leu Thr Ala Met Glu Met Lys His Arg Asn
                  965   970   975

Pro Arg Arg Ala Leu Pro Lys Pro Lys Pro Lys Pro Asn Ala Pro Thr
              980   985   990

Gln Arg Pro Pro Gly Arg Leu Gly Arg Trp Ile Arg Thr Val Ser Asp
      995   1000   1005

Glu Asp Leu Glu
      1010

(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 3261 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION:97..531

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
GGATACGATC GGTCTGACCC CGGGGGAGTC ACCCGGGGAC AGGCCGTCAA GGCCTTGTTC          60


CAGGATGGGA CTCCTCCTTC TACAACGCTA TCATTC GAA GTT AGT TGA GAT CTG          114
                                        Glu Val Ser  *  Asp Leu
                                         1               5


ACA AAC GAT CGC AGC GAT GAC AAA CCT GCA AGA TCA AAC CCA ACA GAT          162
Thr Asn Asp Arg Ser Asp Asp Lys Pro Ala Arg Ser Asn Pro Thr Asp
             10                  15                  20
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2827 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION:112..2745

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
GGATACGATG GGTCTGACCC TCTGGGAGTC ACGAATTAAC GTGGCTACTA GGGGCGATAC          60


CCGCCGCTGG CTGCCACGTT AGTGGCTCCT CTTCTTGATG ATTCTGCCAC C ATG AGT          117
                                                          Met Ser
                                                           1


GAC ATT TTC AAC AGT CCA CAG GCG CGA AGC ACG ATC TCA GCA GCG TTC          165
Asp Ile Phe Asn Ser Pro Gln Ala Arg Ser Thr Ile Ser Ala Ala Phe
         5                   10                  15


GGC ATA AAG CCT ACT GCT GGA CAA GAC GTG GAA GAA CTC TTG ATC CCT          213
```

Gly Ile Lys Pro Thr Ala Gly Gln Asp Val Glu Glu Leu Leu Ile Pro
        20   25   30

5   AAA GTT TGG GTG CCA CCT GAG GAT CCG CTT GCC AGC CCT AGT CGA CTG   261
    Lys Val Trp Val Pro Pro Glu Asp Pro Leu Ala Ser Pro Ser Arg Leu
        35   40   45   50

10 GCA AAG TTC CTC AGA GAG AAC GGC TAC AAA GTT TTG CAG CCG CGG TCT   309
    Ala Lys Phe Leu Arg Glu Asn Gly Tyr Lys Val Leu Gln Pro Arg Ser
                    55   60   65

15 CTG CCC GAG AAT GAG GAG TAT GAG ACC GAC CAA ATA CTC CCA GAC TTA   357
    Leu Pro Glu Asn Glu Glu Tyr Glu Thr Asp Gln Ile Leu Pro Asp Leu
                70   75   80

20 GCA TGG ATG CGA CAG ATA GAA GGG GCT GTT TTA AAA CCC ACT CTA TCT   405
    Ala Trp Met Arg Gln Ile Glu Gly Ala Val Leu Lys Pro Thr Leu Ser
                85   90   95

25 CTC CCT ATT GGA GAT CAG GAG TAC TTC CCA AAG TAC TAC CCA ACA CAT   453
    Leu Pro Ile Gly Asp Gln Glu Tyr Phe Pro Lys Tyr Tyr Pro Thr His
            100   105   110

30 CGC CCT AGC AAG GAG AAG CCC AAT GCG TAC CCG CCA GAC ATC GCA CTA   501
    Arg Pro Ser Lys Glu Lys Pro Asn Ala Tyr Pro Pro Asp Ile Ala Leu
    115   120   125   130

35 CTC AAG CAG ATG ATT TAC CTG TTT CTC CAG GTT CCA GAG GCC AAC GAG   549
    Leu Lys Gln Met Ile Tyr Leu Phe Leu Gln Val Pro Glu Ala Asn Glu
                135   140   145

40 GGC CTA AAG GAT GAA GTA ACC CTC TTG ACC CAA AAC ATA AGG GAC AAG   597
    Gly Leu Lys Asp Glu Val Thr Leu Leu Thr Gln Asn Ile Arg Asp Lys
                150   155   160

45 GCC TAT GGA AGT GGG ACC TAC ATG GGA CAA GCA ACT CGA CTT GTG GCC   645
    Ala Tyr Gly Ser Gly Thr Tyr Met Gly Gln Ala Thr Arg Leu Val Ala
                165   170   175

50 ATG AAG GAG GTC GCC ACT GGA AGA AAC CCA AAC AAG GAT CCT CTA AAG   693
    Met Lys Glu Val Ala Thr Gly Arg Asn Pro Asn Lys Asp Pro Leu Lys
                180   185   190

55 CTT GGG TAC ACT TTT GAG AGC ATC GCG CAG CTA CTT GAC ATC ACA CTA   741
    Leu Gly Tyr Thr Phe Glu Ser Ile Ala Gln Leu Leu Asp Ile Thr Leu
    195   200   205   210

    CCG GTA GGC CCA CCC GGT GAG GAT GAC AAG CCC TGG GTG CCA CTC ACA   789

```
               Pro Val Gly Pro Pro Gly Glu Asp Asp Lys Pro Trp Val Pro Leu Thr
                           215             220             225

  5            AGA GTG CCG TCA CGG ATG TTG GTG CTG ACG GGA GAC GTA GAT GGC GAC        837
               Arg Val Pro Ser Arg Met Leu Val Leu Thr Gly Asp Val Asp Gly Asp
                           230             235             240

 10            TTT GAG GTT GAA GAT TAC CTT CCC AAA ATC AAC CTC AAG TCA TCA AGT        885
               Phe Glu Val Glu Asp Tyr Leu Pro Lys Ile Asn Leu Lys Ser Ser Ser
                           245             250             255

               GGA CTA CCA TAT GTA GGT CGC ACC AAA GGA GAG ACA ATT GGC GAG ATG        933
 15            Gly Leu Pro Tyr Val Gly Arg Thr Lys Gly Glu Thr Ile Gly Glu Met
                           260             265             270

               ATA GCT ATA TCA AAC CAG TTT CTC AGA GAG CTA TCA ACA CTG TTG AAG        981
 20            Ile Ala Ile Ser Asn Gln Phe Leu Arg Glu Leu Ser Thr Leu Leu Lys
                   275             280             285             290

               CAA GGT GCA GGG ACA AAG GGG TCA AAC AAG AAG AAG CTA CTC AGC ATG        1029
 25            Gln Gly Ala Gly Thr Lys Gly Ser Asn Lys Lys Lys Leu Leu Ser Met
                           295             300             305

               TTA AGT GAC TAT TGG TAC TTA TCA TGC GGG CTT TTG TTT CCA AAG GCT        1077
 30            Leu Ser Asp Tyr Trp Tyr Leu Ser Cys Gly Leu Leu Phe Pro Lys Ala
                           310             315             320

               GAA AGG TAC GAC AAA AGT ACA TGG CTC ACC AAG ACC CGG AAC ATA TGG        1125
 35            Glu Arg Tyr Asp Lys Ser Thr Trp Leu Thr Lys Thr Arg Asn Ile Trp
                           325             330             335

               TCA GCT CCA TCC CCA ACA CAC CTC ATG ATC TCC ATG ATC ACC TGG CCC        1173
               Ser Ala Pro Ser Pro Thr His Leu Met Ile Ser Met Ile Thr Trp Pro
 40                        340             345             350

               GTG ATG TCC AAC AGC CCA AAT AAC GTG TTG AAC ATT GAA GGG TGT CCA        1221
               Val Met Ser Asn Ser Pro Asn Asn Val Leu Asn Ile Glu Gly Cys Pro
 45            355             360             365             370

               TCA CTC TAC AAA TTC AAC CCG TTC AGA GGA GGG TTG AAC AGG ATC GTC        1269
               Ser Leu Tyr Lys Phe Asn Pro Phe Arg Gly Gly Leu Asn Arg Ile Val
                           375             380             385

 50            GAG TGG ATA TTG GCC CCG GAA GAA CCC AAG GCT CTT GTA TAT GCG GAC        1317
               Glu Trp Ile Leu Ala Pro Glu Glu Pro Lys Ala Leu Val Tyr Ala Asp
                           390             395             400

 55            AAC ATA TAC ATT GTC CAC TCA AAC ACG TGG TAC TCA ATT GAC CTA GAG        1365
```

```
                Asn Ile Tyr Ile Val His Ser Asn Thr Trp Tyr Ser Ile Asp Leu Glu
                        405                 410                 415

5               AAG GGT GAG GCA AAC TGC ACT CGC CAA CAC ATG CAA GCC GCA ATG TAC        1413
                Lys Gly Glu Ala Asn Cys Thr Arg Gln His Met Gln Ala Ala Met Tyr
                        420                 425                 430

10              TAC ATA CTC ACC AGA GGG TGG TCA GAC AAC GGC GAC CCA ATG TTC AAT        1461
                Tyr Ile Leu Thr Arg Gly Trp Ser Asp Asn Gly Asp Pro Met Phe Asn
                435                 440                 445                 450

15              CAA ACA TGG GCC ACC TTT GCC ATG AAC ATT GCC CCT GCT CTA GTG GTG        1509
                Gln Thr Trp Ala Thr Phe Ala Met Asn Ile Ala Pro Ala Leu Val Val
                                455                 460                 465

20              GAC TCA TCG TGC CTG ATA ATG AAC CTG CAA ATT AAG ACC TAT GGT CAA        1557
                Asp Ser Ser Cys Leu Ile Met Asn Leu Gln Ile Lys Thr Tyr Gly Gln
                                470                 475                 480

25              GGC AGC GGG AAT GCA GCC ACG TTC ATC AAC AAC CAC CTC TTG AGC ACG        1605
                Gly Ser Gly Asn Ala Ala Thr Phe Ile Asn Asn His Leu Leu Ser Thr
                        485                 490                 495

30              CTA GTG CTT GAC CAG TGG AAC TTG ATG AGA CAG CCC AGA CCA GAC AGC        1653
                Leu Val Leu Asp Gln Trp Asn Leu Met Arg Gln Pro Arg Pro Asp Ser
                        500                 505                 510

                GAG GAG TTC AAA TCA ATT GAG GAC AAG CTA GGT ATC AAC TTT AAG ATT        1701
                Glu Glu Phe Lys Ser Ile Glu Asp Lys Leu Gly Ile Asn Phe Lys Ile
35              515                 520                 525                 530

                GAG AGG TCC ATT GAT GAT ATC AGG GGC AAG CTG AGA CAG CTT GTC CTC        1749
                Glu Arg Ser Ile Asp Asp Ile Arg Gly Lys Leu Arg Gln Leu Val Leu
                                535                 540                 545
40
                CTT GCA CAA CCA GGG TAC CTG AGT GGG GGG GTT GAA CCA GAA CAA TCC        1797
                Leu Ala Gln Pro Gly Tyr Leu Ser Gly Gly Val Glu Pro Glu Gln Ser
                                550                 555                 560
45
                AGC CCA ACT GTT GAG CTT GAC CTA CTA GGG TGG TCA GCT ACA TAC AGC        1845
                Ser Pro Thr Val Glu Leu Asp Leu Leu Gly Trp Ser Ala Thr Tyr Ser
                                565                 570                 575

50              AAA GAT CTC GGG ATC TAT GTG CCG GTG CTT GAC AAG GAA CGC CTA TTT        1893
                Lys Asp Leu Gly Ile Tyr Val Pro Val Leu Asp Lys Glu Arg Leu Phe
                        580                 585                 590

55              TGT TCT GCT GCG TAT CCC AAG GGA GTA GAG AAC AAG AGT CTC AAG TCC        1941
```

```
                Cys Ser Ala Ala Tyr Pro Lys Gly Val Glu Asn Lys Ser Leu Lys Ser
                595                 600             ·605              610
```

```
                AAA GTC GGG ATC GAG CAG GCA TAC AAG GTA GTC AGG TAT GAG GCG TTG        1989
                Lys Val Gly Ile Glu Gln Ala Tyr Lys Val Val Arg Tyr Glu Ala Leu
                                    615             620             625
```

```
                AGG TTG GTA GGT GGT TGG AAC TAC CCA CTC CTG AAC AAA GCC TGC AAG        2037
                Arg Leu Val Gly Gly Trp Asn Tyr Pro Leu Leu Asn Lys Ala Cys Lys
                                630             635             640
```

```
                AAT AAC GCA GGC GCC GCT CGG CGG CAT CTG GAG GCC AAG GGG TTC CCA        2085
                Asn Asn Ala Gly Ala Ala Arg Arg His Leu Glu Ala Lys Gly Phe Pro
                                645             650             655
```

```
                CTC GAC GAG TTC CTA GCC GAG TGG TCT GAG CTG TCA GAG TTC GGT GAG        2133
                Leu Asp Glu Phe Leu Ala Glu Trp Ser Glu Leu Ser Glu Phe Gly Glu
                                660             665             670
```

```
                GCC TTC GAA GGC TTC AAT ATC AAG CTG ACC GTA ACA TCT GAG AGC CTA        2181
                Ala Phe Glu Gly Phe Asn Ile Lys Leu Thr Val Thr Ser Glu Ser Leu
                675                 680             685             690
```

```
                GCC GAA CTG AAC AAG CCA GTA CCC CCC AAG CCC CCA AAT GTC AAC AGA        2229
                Ala Glu Leu Asn Lys Pro Val Pro Pro Lys Pro Pro Asn Val Asn Arg
                                695             700             705
```

```
                CCA GTC AAC ACT GGG GGA CTC AAG GCA GTC AGC AAC GCC CTC AAG ACC        2277
                Pro Val Asn Thr Gly Gly Leu Lys Ala Val Ser Asn Ala Leu Lys Thr
                                710             715             720
```

```
                GGT CGG TAC AGG AAC GAA GCC GGA CTG AGT GGT CTC GTC CTT CTA GCC        2325
                Gly Arg Tyr Arg Asn Glu Ala Gly Leu Ser Gly Leu Val Leu Leu Ala
                                725             730             735
```

```
                ACA GCA AGA AGC CGT CTG CAA GAT GCA GTT AAG GCC AAG GCA GAA GCC        2373
                Thr Ala Arg Ser Arg Leu Gln Asp Ala Val Lys Ala Lys Ala Glu Ala
                                740             745             750
```

```
                GAG AAA CTC CAC AAG TCC AAG CCA GAC GAC CCC GAT GCA GAC TGG TTC        2421
                Glu Lys Leu His Lys Ser Lys Pro Asp Asp Pro Asp Ala Asp Trp Phe
                755                 760             765             770
```

```
                GAA AGA TCA GAA ACT CTG TCA GAC CTT CTG GAG AAA GCC GAC ATC GCC        2469
                Glu Arg Ser Glu Thr Leu Ser Asp Leu Leu Glu Lys Ala Asp Ile Ala
                                775             780             785
```

```
                AGC AAG GTC GCC CAC TCA GCA CTC GTG GAA ACA AGC GAC GCC CTT GAA        2517
```

```
        Ser Lys Val Ala His Ser Ala Leu Val Glu Thr Ser Asp Ala Leu Glu
                    790             795             800
```

```
        GCA GTT CAG TCG ACT TCC GTG TAC ACC CCC AAG TAC CCA GAA GTC AAG        2565
        Ala Val Gln Ser Thr Ser Val Tyr Thr Pro Lys Tyr Pro Glu Val Lys
                    805             810             815
```

```
        AAC CCA CAG ACC GCC TCC AAC CCC GTT GTT GGG CTC CAC CTG CCC GCC        2613
        Asn Pro Gln Thr Ala Ser Asn Pro Val Val Gly Leu His Leu Pro Ala
                    820     .       825             830
```

```
        AAG AGA GCC ACC GGT GTC CAG GCC GCT CTT CTC GGA GCA GGA ACG AGC        2661
        Lys Arg Ala Thr Gly Val Gln Ala Ala Leu Leu Gly Ala Gly Thr Ser
        835             840             845             850
```

```
        AGA CCA ATG GGG ATG GAG GCC CCA ACA CGG TCC AAG AAC GCC GTG AAA        2709
        Arg Pro Met Gly Met Glu Ala Pro Thr Arg Ser Lys Asn Ala Val Lys
                        855             860             865
```

```
        ATG GCC AAA CGG CGG CAA CGC CAA AAG GAG AGC CGC TAACAGCCAT             2755
        Met Ala Lys Arg Arg Gln Arg Gln Lys Glu Ser Arg
                    870             875
```

```
        GATGGGAACC ACTCAAGAAG AGGACACTAA TCCCAGACCC CGTATCCCCG GCCTTCGCCT      2815
```

```
        GCGGGGGCCC CC                                                         2827
```

Claims

1. A birnavirus mutant which is not able to produce a native VP5 protein as a result of a mutation in the VP5 gene of the birnavirus genome, **characterised in that** the mutation comprises:

   (i) a substitution of at least two nucleotides of the start codon of the VP5 gene, and
   (ii) a stop codon in each of the three open reading frames in the 5'-end of the VP5 gene.

2. A birnavirus mutant according to claim 2, **characterised in that** the birnavirus is infectious bursal disease virus (IBDV).

3. A birnavirus mutant according to claims 1-2, **characterised in that** the mutation is in the genome of a virulent field virus.

4. A birnavirus mutant according to claim 2, **characterised in that** the mutation is in the genome of a vaccine strain, preferably in vaccine strain D78.

5. A birnavirus mutant according to claims 2-4, **characterised in that** the mutant has a mutated start codon and three stop codons in the 5'-end of the VP5 gene as shown in SEQ ID No: 7.

6. A birnavirus mutant according to claims 2-5, **characterised in that** the IBDV expresses a chimeric VP2 protein

comprising virus neutralising epitopes of different antigenic IBDV types.

7. A vaccine against a birnavirus infection in animals, **characterised in that** it comprises a birnavirus mutant according to claims 1-6 and a pharmaceutically acceptable carrier.

8. A method for the attenuation of virulence of a birnavirus in an animal, comprising the step of introducing a mutation in the VP5 gene as a result of which the birnavirus is not able to produce a VP5 protein.

9. A method according to claim 9, wherein the mutation is a substitution.

10. A method according to claims 8-9, wherein the birnavirus is infectious bursal disease virus (IBDV).

11. A method according to claims 8-10, wherein the mutation is in the genome of a virulent field virus.

12. A method according to claims 8-11, wherein the mutation comprises a substitution of at least two nucleotides of the start codon of the VP5 gene.

13. A method according to claim 12 wherein the mutation comprises additionally one or more stop codons in the 5'-end of the VP5 gene.

14. A method according to claim 13, wherein the mutation comprises a stop codon in each of the three open reading frames.

15. A method according to claim 14, wherein the mutation is in the start codon and comprises three stop codons in the 5'-end of the VP5 gene as shown in SEQ ID No: 7.

Patentansprüche

1. Eine Birnavirus Mutante, die aufgrund einer Mutation im VP5 Gen des Birnavirus Genoms kein natives VP5 Protein produzieren kann, **dadurch gekennzeichnet, dass** die Mutation:

   (i) eine Substitution von mindestens zwei Nukleotiden des Startcodons des VP5 Gens, und

   (ii) ein Stopcodon in jedem der drei offenen Leserahmen am 5'-Ende des VP5 Gens.

   umfasst.

2. Eine Birnavirus Mutante gemäss Anspruch 1, **dadurch gekennzeichnet dass** das Birnavirus ein Infektiöses Bursitis Virus (IBDV) darstellt.

3. Eine Birnavirus Mutante gemäss Ansprüchen 1-2, **dadurch gekennzeichnet, dass** die Mutation im Genom eines virulenten Feldvirus ist.

4. Eine Birnavirus Mutante gemäss Anspruch 2, **dadurch gekennzeichnet, dass** die Mutation im Genom eines Impfstoff-Stammes, vorzugsweise im Impfstoff-Stamm D78, ist.

5. Eine Birnavirus Mutante gemäss Ansprüchen 2-4, **dadurch gekennzeichnet, dass** die Mutante ein mutiertes Startcodon und drei Stopcodons am 5'-Ende des VP5-Gens wie in SEQ ID No:7 dargestellt besitzt.

6. Eine Birnavirus Mutante gemäss Ansprüchen 2-5, **dadurch gekennzeichnet, dass** das IBDV ein chimäres VP2 Protein exprimiert, das Virus-neutralisierende Epitope von unterschiedlichen, antigenen IBDV-Typen umfasst.

7. Ein Impfstoff gegen eine Birnavirus Infektion in Tieren, **dadurch gekennzeichnet, dass** er eine Birnavirus Mutante gemäss Ansprüchen 1-6 und einen pharmazeutisch verträglichen Träger umfasst.

8. Verfahren zur Abschwächung der Virulenz eines Birnavirus in einem Tier, welches den Schritt des Einführens einer Mutation in das VP5 Gen umfasst, als Folge dessen das Birnavirus kein VP5 Protein produzieren kann.

9. Verfahren gemäss Anspruch 8, worin die Mutation eine Substitution darstellt.

10. Verfahren gemäss Ansprüchen 8-9, worin das Birnavirus ein Infektiöses Bursitis Virus (IBDV) darstellt.

11. Verfahren gemäss Ansprüchen 8-10, worin die Mutation im Genom eines virulenten Feldvirus ist.

12. Verfahren gemäss Ansprüchen 8-11, worin die Mutation eine Substitution von mindestens zwei Nukleotiden des Startcodons des VP5 Gens umfasst.

13. Verfahren gemäss Anspruch 12, worin die Mutation zusätzlich ein oder mehrere Stopcodons am 5'-Ende des VP5-Gens umfasst.

14. Verfahren gemäss Anspruch 13, worin die Mutation in jedem der drei offenen Leserahmen ein Stopcodon umfasst.

15. Verfahren gemäss Anspruch 14, worin die Mutation im Startcodon ist und drei Stopcodons am 5'-Ende des VP5-Gens wie in SEQ ID No:7 dargestellt umfasst.

Revendications

1. Un mutant du birnavirus qui est incapable de produire une protéine VP5 native, résultant d'une mutation dans le gène VP5 du génome du birnavirus, **caractérisé en ce que** la mutation comprend :

   i) une substitution d'au moins deux nucléotides du codon de départ du gène VP5, et
   ii) un codon d'arrêt dans chacun des trois cadres ouverts de lecture dans l'extrémité 5' du gène VP5.

2. Un mutant du birnavirus selon la revendication 1, **caractérisé en ce que** le birnavirus est le virus de la bursite infectieuse aviaire (IBDV : *Infections Bursal Diseuse Virus*).

3. Un mutant du birnavirus selon la revendication 1 ou 2, **caractérisé en ce que** la mutation est dans le génome d'un virus sauvage virulent.

4. Un mutant du birnavirus selon la revendication 2, **caractérisé en ce que** la mutation est dans le génome d'une souche de vaccin, de préférence dans la souche de vaccin D78.

5. Un mutant du birnavirus selon l'une des revendications 2 à 4, **caractérisé en ce que** le mutant a un codon de départ ayant subi une mutation et les trois codons d'arrêt dans l'extrémité 5' du gène VP5 tel que représenté dans la SEQ ID n° 7.

6. Un mutant du birnavirus selon l'une des revendications 2 à 5, **caractérisé en ce que** l'IBDV exprime une protéine chimère VP2 comprenant des épitopes neutralisant des virus de différents types antigènes d'IBDV.

7. Un vaccin contre une infection par le birnavirus chez des animaux, **caractérisé en ce qu'**il comprend un mutant du birnavirus selon l'une des revendications 1 à 6 et un support pharmaceutiquement acceptable.

8. Un procédé pour atténuer la virulence d'un birnavirus chez un animal, comprenant l'étape consistant à introduire une mutation dans le gène VP5 pour que le birnavirus soit incapable de produire une protéine VP5.

9. Un procédé selon la revendication 9, dans lequel la mutation est une substitution.

10. Un procédé selon la revendication 8 ou 9, dans lequel le birnavirus est le virus de la bursite infectieuse aviaire (IBDV).

11. Un procédé selon l'une des revendications 8 à 10, dans lequel la mutation est dans le génome d'un virus sauvage virulent.

12. Un procédé selon l'une des revendications 8 à 11, dans lequel la mutation comprend une substitution d'au moins deux nucléotides du codon de départ du gène VP5.

13. Un procédé selon la revendication 12, dans lequel la mutation comprend en outre un ou plusieurs codons d'arrêt dans l'extrémité 5' du gène VP5.

14. Un procédé selon la revendication 13, dans lequel la mutation comprend un codon d'arrêt dans chacun des trois cadres ouverts de lecture.

15. Un procédé selon la revendication 14, dans lequel la mutation est située dans le codon de départ et comprend trois codons d'arrêt dans l'extrémité 5' du gène VP5 tel que représenté dans la SEQ ID n° 7.

Figure 1

Genomic organization of segment A of strain D78 and segment B of strain P2
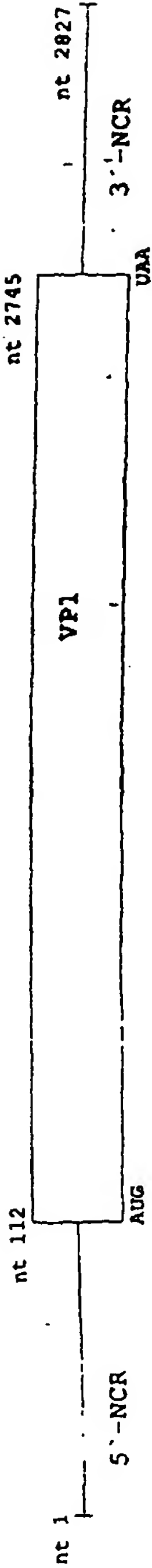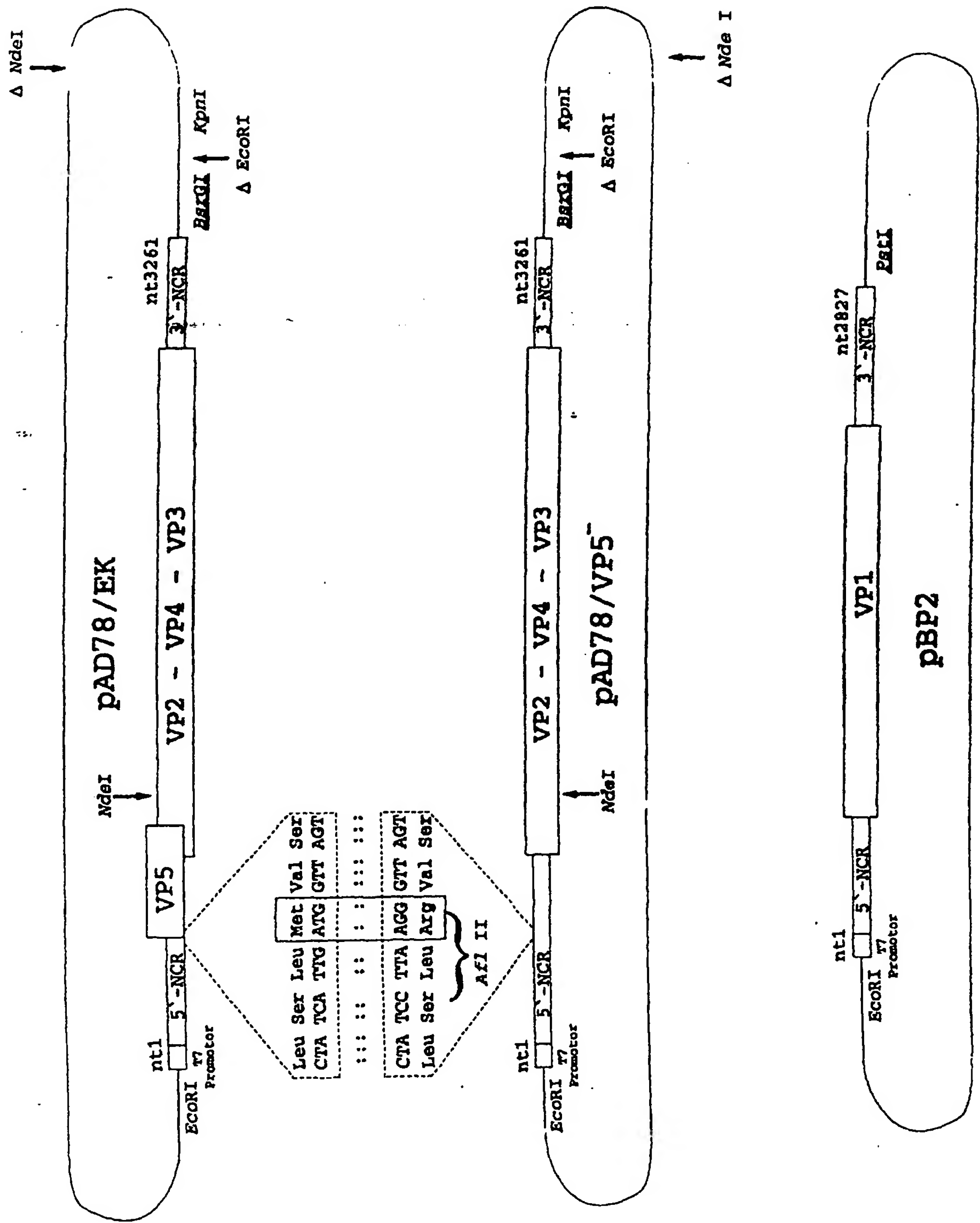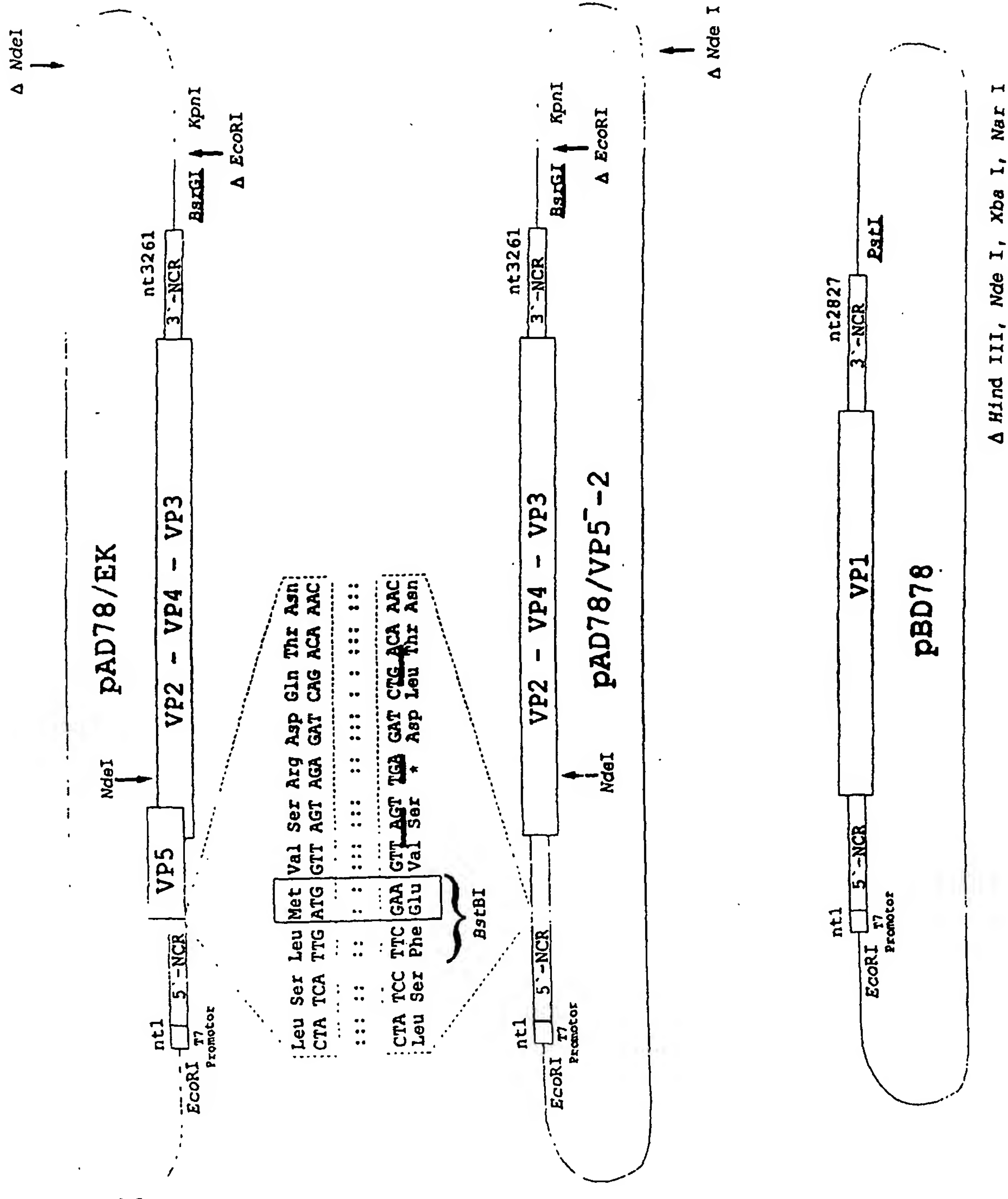
D78 segment A
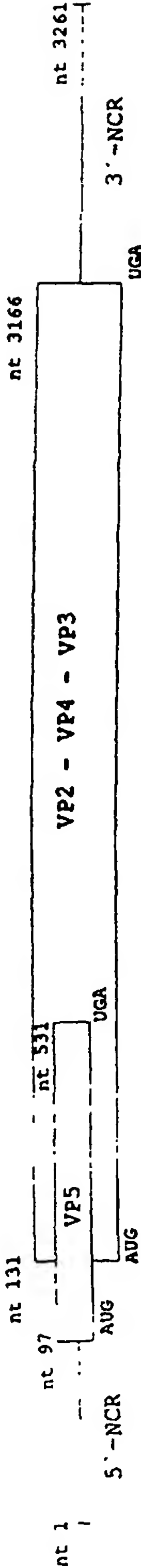
P2 segment B

Figure 2

Figure 3

Figure 4

Figure 5